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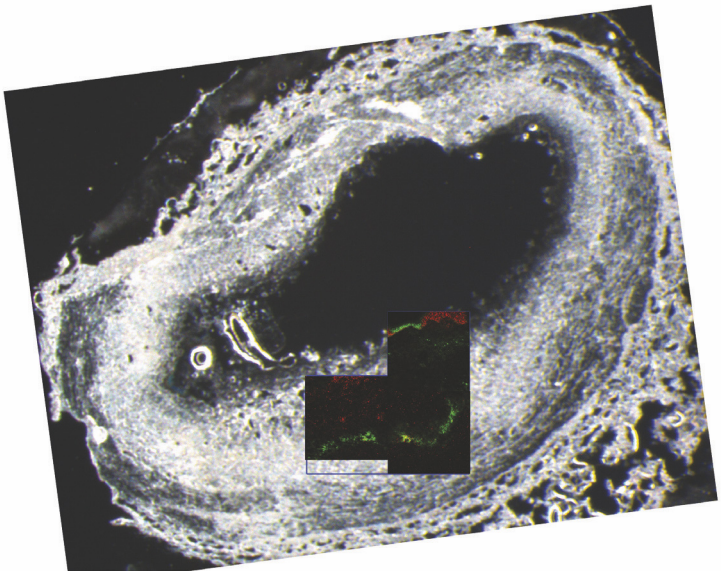
SATU LEHTI

Extracellular Lipid Particles in Atherosclerosis and Aortic Stenosis



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DEPARTMENT OF BIOSCIENCES
FACULTY OF BIOLOGICAL AND ENVIRONMENTAL SCIENCES
DOCTORAL PROGRAMME IN INTEGRATIVE LIFE SCIENCE
UNIVERSITY OF HELSINKI

Extracellular lipid particles in atherosclerosis and aortic stenosis

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ACADEMIC DISSERTATION

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Life is science and science is life.

To my family and to the friends not among us anymore.

Contents

List of original publications

Study I
Lehti S., Sjövall P., Käkelä R., Mäyränpää M.I., Kovanen P.T., Öörni K. (2015)
Spatial distributions of lipids in atherosclerosis of human coronary arteries studied by time-of-flight secondary ion mass spectrometry. Am J Pathol 185: 1216-1233

Study II
Lehti, S. Käkelä, R, Hörkkö, S., Kummu, O., Helske-Suihko, S., Kupari, M., Werkkala, K., Kovanen, P. T., and Öörni, K. (2013)
Modified lipoprotein-derived lipid particles accumulate in human stenotic aortic valves. PLoS ONE 8(6): e65810. doi:10.1371/journal.pone.0065810

Study III
Lehti, S., Nguyen, S.D., Belevich, I., Vihinen, H., Heikkilä, H-M., Soliymani, R., Käkelä, R., Saksi, J., Jauhiainen, M., Grabowski, G.A., Kummu, O., Hörkkö, S., Baumann. M., Lindsberg, P.J., Jokitalo, E., Kovanen, P.T., and Öörni, K.
Extracellular lipid accumulates in human carotid arteries as distinct three-dimensional structures with proinflammatory properties (Submitted)

Author's contribution

I The author participated in the design of the study and individual experiments, analyzed the data of TOF-SIMS-analyses, designed and analyzed the immunohistochemical stainings and performed the statistical analysis. The author interpreted the results of experiments and participated in writing and editing the manuscript.

II The author participated in the design of individual experiments. The author isolated the extracellular lipid droplets from the aortic valve leaflets and fractionated the lipid particles, performed the cholesterol-concentration assays and ELISA-analysis of the lipid particles, performed the Western blot-analysis, extracted the lipids and performed the mass spectrometry (100 %) and thin layer chromatography (50 %) -analyses of the lipids. The author performed the statistical analysis of the data, analyzed the data, interpreted the data. and participated in writing and editing the manuscript.

III The author participated in the design of the study and individual experiments. The author isolated the extracellular lipid particles and fractionated the particles, extracted the lipids and performed the mass spectrometry analyses, prepared the cholesterol crystals from the extracellular lipid particles, performed the cell experiments, Western Blot-analyses, and participated in the ELISA-analyses and PCR-analyses. The author prepared the tissues, cut the sections and performed the immunohistochemistry, and fixed the samples for electron microscopy. The author participated in the CD-analysis. The author analyzed and interpreted the data, performed the statistical analyses and participated in writing and editing the manuscript.

List of abbreviations

4-HNE	4-Hydroxynonenal
ABCA1	ATP-binding cassette A1
ABCG1	ATP-binding cassette G1
ACAT	Acty-coA:cholesterol acyltransferase
ALA	α-linolenic acid
ANGPTL	Angiopoietin-like protein
ApoA	Apolipoprotein A
ApoB	Apolipoprotein B
ApoC	Apolipoprotein C
ApoE	Apolipoprotein E
CCA	Common carotid artery
CD	Circular dichroism
CD36	Cluster of differentiation 36
CE	Cholesteryl ester
CETP	Cholesteryl ester transfer protein
Chol	Cholesterol
D_2O	Deuterium oxide
DAMP	Damage-associated molecular pattern
ECA	Extenal carotid artery
E-LDL	Enzymatically modified LDL
EM	Electron microscopy
ESI-MS	Electrospray ionization mass spectrometry
ET	Electron tomography
GLA	γ-linolenic acid
HDL	High density lipoprotein
HMG-CoA reductase	Hydroxymethyl-glutaryl-coenzymeA reductase
ICA	Internal carotid artery
IDL	Intermediate density lipoprotein
IEL	Internal elastic lamina
IL-1β	Interleukin 1β
LCAT	Lecithin-cholesterol acyltransferase
LDL	Low density lipoprotein
LOX	Lectin-like oxidized LDL receptor
LPC	Lysophosphatidylcholine
LPL	Lipoprotein lipase
MAA	Malondialdehyde-acetaldehyde
MALDI	Matrix assisted laser desortption/ionization
MDA	Malondialdehyde
MMP	Matrix metalloproteinase
NLRP	Nucleotide-binding domain leucine-rich repeat containing protein
NMR	Nuclear magnetic resonance
Ox-LDL	Oxidized LDL
PAMP	Pathogen-associated molecular pattern
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine

PI	Phosphatidylinositol
PL	Phospholipid
PLA_2	Phospholipase A_2
PS	Phosphatidylserine
SAA	Serum amyloid A
SEM	Scanning electron microscopy
SIMS	Secondary ion mass spectrometry
SM	Sphingomyelin
SMase	Sphingomyelinase
SR-A	Scavenger receptor class A
SR-BI	Scavenger receptor class B type I
TAG	Triacylglycerol
TC	Total cholesterol
TEM	Transmission electron microscopy
TLC	Thin layer choromatography
TLR	Toll like receptor
TOF-SIMS	Time of flight secondary ion mass spectrometry
UC	Unesterified cholesterol
VLDL	Very low density lipoprotein

Abstract

Aortic stenosis and athrosclerosis are slowly progressing diseases. Being clinically silent, as they start developing decades before they cause symptoms, cardiovascular diseases and atherosclerosis in particular, are the leading cause of morbitidy and mortality in the Western world. Lipid accumulation begins both in the artery walls and in the aortic valves before any clinical signs of atherosclerosis or aortic stenosis can be detected. While atherosclerotic lesions are characterized with cells filled with lipid droplets i.e. foam cells, and they are found to be calcified only in the late stages of atherosclerosis, the stenotic aortic valve leaflets contain both lipid droplets and calcified nodules already in early lesions. The proteoglycan matrix common to artery wall and aortic valve leaflets retains the entering lipoprotein particles that are then enzymatically and oxidatively modified. Such modifications have an ability to transform the non-inflammatory plasma lipoprotein particles into crystals and particles that can induce sterile inflammation in the various intimal and valvular cells.

This thesis was set to study the distribution and characteristics of the extracellular lipid and to reveal the origin of the extracellular lipid particles. Prior to the analyses of the isolated extracellular lipid particles, the human carotid artery plaques were imaged with three-dimensional electron microscopy and sections of human coronary arteries were analyzed with imaging mass spectrometry to study the spatial distribution of lipids in different stages of atherosclerosis. In the human coronary arteries, the lipid domains found in advanced atherosclerotic lesions were different from the domains found in atheroma-stage artery sections, and even more different from healthy sections of coronary arteries. In the human carotid artery plaques, cholesterol crystals were found to be large sheets or needle-like structures, and they appeared to be growing out from large lipid particles in the intima. For the purpose of studying the chemical and physical characteristics of the extracellular lipid particles, the extracellular particles were isolated from aortic valve leaflets and coronary artery plaques. The lipid particles were examined with multiple tools to study their lipid composition, protein composition, protein structure, size, and density. The extracellular lipid particles, both in human aortic valve leaflets and human carotid arteries, were found to be derived from plasma lipoproteins, mainly from low density lipoprotein (LDL) or very low density lipoprotein (VLDL). Apart from multiple small exchangeable lipoproteins, like apolipoprotein (apo) E, apoA-family, and apoC-family, the particles contained mainly apolipoprotein B-100 (apoB-100), the integral protein of LDL and VLDL, and they contained features which suggest that they were multiply modified. Another goal was to find which modifications would change the intimal or valvular extracellular lipid particles to such fused and aggregated lipid particles that were found in the valves and intimal plaques, and which modifications would induce a sterile inflammatory response similar to the response the extracellular lipid particles induce. To study the possible culprit modifications, both the extracellular lipid particles, in vitro-generated cholesterol crystals, and LDL that was modified by lipolysis, proteolysis, and oxidation, were applied to human primary monocyte-derived macrophages. Both the isolated extracellular lipid particles and LDL modified with a combination of phospholipolysis by PLA_2 and cholesterol esterase were found to be able to activate a multiprotein complex inflammasome in human primary monocyte-derived macrophages *in vitro*, and to induce the secretion of proinflammatory cytokines.

According to the results of this thesis, the lipid particles in the arterial intima and in the aortic valve are active components of atherosclerosis and aortic stenosis. They can induce the cells in the intima and in the valve to produce inflammatory cytokines and thus can affect the progress of these diseases.

Tiivistelmä

Aorttastenoosi eli aorttaläpän kalkkinen kovettumatauti ja ateroskleroosi eli valtimokovettumatauti ovat hitaasti eteneviä tauteja. Koska nämä sydän- ja verisuonitaudit kehittyvät piilossa oireita aiheuttamatta jopa vuosikymmenten ajan, ne ovat yhä edelleen suomalaisten ja läntisen maailman kuolinsyytilastojen kärjessä. Lipidit eli rasva-aineet alkavat kertyä sekä aorttaläppään että valtimoiden seinämään jo paljon ennen kuin mitään kliinisiä merkkejä on havaittavissa. Ateroskleroosissa plakit ovat tyypillisesti täynnä rasvapartikkeleita eli lipidipartikkeleita ja soluja, jotka ovat täynnä rasvapisaroita (vaahtosoluja) ja ne kalkkiintuvat vasta taudin myöhäisvaiheessa. Aorttaläpissä on sen sijaan yhtä aikaa sekä lipidipartikkeleita ja kiteytynyttä kalkkia. Sekä aorttaläpässä että valtimon seinämässä on proteoglykaanikerros, johon lipidipartikkelit tarttuvat kiinni. Kiinni tarttuneet lipidipartikkelit voivat voivat muokkaantua entsyymien vaikutuksesta tai hapettumisen seurauksena. lipidipartikkelien muokkaantuminen voi saada aorttaläpän tai valtimon sisäkerroksen solut reagoimaan steriilillä tulehdusreaktiolla tai muokkaantuneet lipidipartikkelien sisältämä kolesteroli voi kiteytyä suuriksi kolesterolikiteiksi.

Tässä väitöskirjassa tutkittiin solunulkoisten lipidipartikkeleiden ominaisuuksia ja niiden levittäytymistä solujen väliseen tilaan. Siten pyrittiin selvittämään solunulkoisten lipidipartikkeleiden alkuperä. Ihmisen kaulavaltimoiden seinämästä valmistettiin malleja kolmiulotteisten elektronimikroskooppikuvien avulla ja ihmisen sepelvaltimoleikkeitä tutkittiin kuvantavalla massaspektrometrilla. Näin saatiin selville, kuinka lipidipartikkelit sijaitsivat valtimoiden seinämässä. Terveiden sepelvaltimoiden lipidipartikkelikertymien laatu ja paikka erosivat aterooma-vaiheen plakkien lipidipartikkelikertymistä ja vielä enemmän ne erosivat pitkälle edenneestä ateroskleroottisesta plakista. Ihmisen kaulavaltimoiden plakeissa kolesterolikiteet olivat sekä neulamaisia että suuria levyjä ja ne näyttivät kasvavan suoraan suurista lipidipartikkeleista. Kemiallisten ja fysikaalisten ominaisuuksien selvittämiseksi lipidipartikkelit eristettiin sekä aorttaläpistä että kaulavaltimoista. Niistä tutkittiin lipidikoostumusta, proteiinikoostumusta, proteiiniosan rakennetta, partikkelien kokoa ja tiheyttä. Sekä aorttaläpän että kaulavaltimon lipidipartikkeleiden havaittiin olevan peräisin plasman apolipoproteiini (apo)B-100:aa sisältävistä lipoproteiinipartikkeleista, todennäköisimmin joko kevyen lipoproteiinin (LDL) tai erittäin kevyen lipoproteiinin (VLDL) partikkeleista. Partikkelit sisälsivät apoB-100:n lisäksi myös pieniä vaihdettavia apolipoproteiineja, kuten apolipoproteiini (apo) E:tä, apoA-proteiiniperheen ja apoC-proteiiniperheen apolipoproteiineja. ApoB-100 (LDL:n ja VLDL:n tärkein rakenneproteiini) oli yleisin löydetty proteiini. Lipidipartikkeleissa nähtiin merkkejä moninkertaisesta muokkautumisesta.

Tutkimuksen toisena tavoitteena oli saada selville, miten aorttaläpän ja valtimon sisäseinämään jääneet lipidipartikkelit muuntuvat plasman lipoproteiineista suuriksi fuusioituneiksi ja aggregoituneiksi lipidipartikkeleiksi. Lisäksi haluttiin tietää, mitkä muokkausmekanismit muuntaisivat lipoproteiinit niin, että solut reagoisivat steriilillä tulehdusreaktiolla eli inflammasomi-kompleksin aktivaatiolla. Mahdollisia muuntumismekanismeja tutkittiin muokkaamalla LDL-partikkeleita lipolyyttisillä ja proteolyyttisillä entsyymeillä ja hapettamalla niitä. Näin muokattuja LDL-partikkeleita ja kaulavaltimoista eristettyjä lipidipartikkeleita annettiin ihmisen plasmasta eristetyistä monosyyteistä erilaistetuille makrofageille. Sekä solunulkoiset lipidipartikkelit että fosfolipolyysillä muokatut plasman lipoproteiinit saivat koeolosuhteissa makrofagit reagoimaan inflammasomin aktivaatiolla ja tuottamaan tulehdusvälittäjäaineita.

Väitöskirjan tulosten mukaan voidaan todeta, että lipidipartikkelit aorttaläpässä ja valtimon sisäseinämässä ovat oleellinen osa aorttastenoosin ja ateroskleroosin syntyprosessissa. Ne saavat aorttaläpän ja valtimon sisäseinämän solut tuottamaan tulehdusvälittäjäaineita ja vaikuttavat siten sekä aorttastenoosin että ateroskleroosin etenemiseen.

Introduction

Cardiovascular diseases continue to be the largest cause of morbidity and mortality in Western world. Since 1950's, the mortality rates to cardiovascular diseases have been declining (WHO, 2010). Still, since the recognition of cholesterol as one of the main culprits, serum cholesterol levels have declined only slightly (Farzadfar et al., 2011), but cardiovascular diseases remain the major cause for morbidity (WHO, 2015). As the process in the arteries leading to atherosclerosis may be more complex than the simple accumulation of cholesterol-containing particles, we still have several details to clear.

Atherosclerosis and aortic stenosis are two different diseases in the same system, yet the major risk factors of both diseases are shared: smoking is found to worsen both conditions, old age, and male sex, or rather lack of estrogen, hyperlipidemia, hypertension, and diabetes are in the long list of risk factors of both diseases (Hansson, 2005; Lindroos et al., 1994; Rajamannan, 2009). Both diseases are also linked with lipid accumulation in the extracellular matrix. While cholesterol is considered the most important risk factor in both atherosclerosis and aortic valve disease, lowering of plasma cholesterol with statins alleviates atherosclerosis, but has no significant effect regarding progression of aortic stenosis (Rossebo et al., 2008). In atherosclerosis, if plasma cholesterol is lowered to a level low enough (by 1 mmol/l), already developed plaques start to regress (Puri et al., 2015). In contrast to that, already stenotic aortic valves do not become less stenotic even if the plasma cholesterol level is intensively lowered with combined use of a statin and ezetimibe (Rossebo et al., 2008).

Both in atherosclerosis and aortic stenosisthe disease initiates when cholesterol-containing lipoproteins accumulate in the subendothelial extracellular matrix of the arterial system, i.e. in the subendothelial matrix of the arterial intima or of the aortic valve, respectively. Arteries as well as aortic valves contain proteoglycans (Della Rocca et al., 2000; Wight and Ross, 1975a; Wight and Ross, 1975b), that act as cushions against pressure surges of the blood flow. In this dense proteoglycan matrix lipoproteins attach and accumulate (Williams and Tabas, 1995) as extracellular lipid particles (Guyton and Klemp, 1989; Pasquinelli et al., 1989). The retained lipoproteins can become modified by the lipolytic, proteolytic, and oxidative enzymes and agents that the local cells secrete, and the modified lipoproteins can then be avidly taken up by macrophages to form lipid-filled foam cells. The modified lipid particles are also a source of cholesterol crystals, which are a recognized activator of a multiprotein complex called inflammasome (Rajamäki et al., 2010). Inflammasome is activated by several different factors, such as by the aforementioned cholesterol crystals, as well as by monosodium urate crystals (Martinon et al., 2006), asbestos (Sayan and Mossman, 2016), extremely fine carbon particles (Murphy et al., 2012) acid pH (Rajamäki et al., 2013) and the acute phase protein SAA (Niemi et al., 2011).

The aim of this thesis work was to characterize the extracellular lipoproteins and lipid particles in human atherosclerotic arterial intima and in human stenotic aortic valves, and to compare them with plasma lipoproteins and the lipids contained in them. This study also aimed to study different modifications which have affected the lipoprotein particles, and to find how and why extracellular lipid particles have been formed, and what kind of an effect the extracellular lipid particles may have on atherosclerosis. The spatial distribution of lipids in different stages of atherosclerosis was also studied together with the microstructure of the extracellular lipid particles and cholesterol crystals in a carotid artery plaque. As cholesterol crystals have been found to be a strong inflammasome activator in human (Rajamäki et al., 2010) and mouse (Duewell et al., 2010) macrophages, also the effect of extracellular lipid particles on human primary macrophages was studied.

Review of the literature

Normal aortic valve and arterial wall

Aortic valve

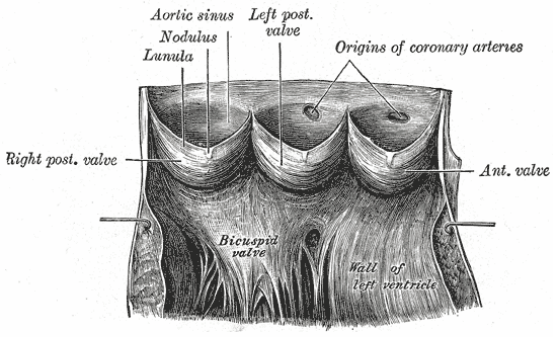

Figure 1. Aortic valve. Aortic valve consist of three cusps that are supported by a connective tissue ring, annulus. Coronary arteries have their origins from two of the cusps. (Gray, 1918), plate 497. (Schönherr et al.) (PD-Art) Public Domain

Aortic valve (Figure 1) is one of two semilunar valves of the heart, and is situated in the aortic root, between left ventricle and aorta, and it is attached to connective tissue ring, annulus (Misfeld and Sievers, 2007). The valve is normally formed of three cusps, but occasionally only two cusps are found. Rarely one or four, or even five cusps are found (Roberts and Ko, 2005; Schmidt et al., 2008). On the aortic side, the cusps and aortic root form sinus of Valsalva, a bulging structure in the beginning of aorta, from where the coronary arteries begin. Aortic valve prevents reflux of blood after the heart has pumped blood into the aorta, maintaining one-way flow of blood (Misfeld and Sievers, 2007).

Valvular spongiosa, ventricularis, and fibrosa

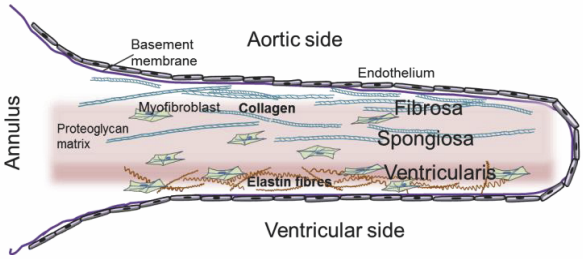

Figure 2. A schematic drawing of a healthy aortic valve. Aortic valve consists of endothelium and three layers: fibrosa (on the aortic side), spongiosa, and ventricularis (on the ventricular side of the valve).

Aortic valve leaflets are extensions of endocardium and formed from an endocardial fold (de Lange et al., 2004; Hinton et al., 2006), covered by endothelium on its both sides. Endothelium of the valve differs from endothelium of an artery (Deck, 1986). In the valve, endothelial cells are aligned perpendicularly against blood flow, and they produce vasoactive substances that bring the myofibroblasts of the valve to contract when the endothelium is intact (Deck, 1986). Three separate layers can be found in the aortic valves leaflets:

fibrosa on the aortic side, spongiosa in the middle, and on the ventricular side of the valve, ventricularis (Figure 2). Fibrosa, has collagen fibers that are perpendicular to the elastin fibers of the ventricularis (Scott and Vesely, 1995) They offer support to the valve leaflets, as blood flow on the aortic side is turbulent. Fibrosa consists of myofibroblasts that contain both vimentin and smooth muscle actin (Della Rocca et al., 2000). (Latif et al., 2005; Stephens, Chu, and Grande-Allen, 2008). Myofibroblasts secrete collagen, and proteoglycans, which will be discussed in a later chapterpage 19. Fibrosa can be as thin as some cell layers. Spongiosa, the second layer in between ventricularis and fibrosa, is sponge-like and soft, containing fibroblasts, mesenchymal cells, and extracellular matrix (Della Rocca et al., 2000). Ventricularis consists of fibroblasts and elastin fibers the cells secrete (Della Rocca et al., 2000), and valvular interstitial cells (VIC) that have both fibroblast and smooth muscle cell features (Filip, Radu, and Simionescu, 1986). Ventricularis has also proteoglycans to even the pressure and stretch due to blood surge against the valve, and the total proteoglycan content is higher than in spongiosa or fibrosa (Stephens, Chu, and Grande-Allen, 2008). VICs are found also in other layers, as they are arranged in net-like structure around valvular leaflets.

Arteries

Arteries are blood vessels that carry oxygenated blood from the heart to the periphery (Figure 2). As the heart creates varying pressure conditions in aorta and in the largest arteries, they require special adaptations. Arterial walls of the aorta and largest arteries receive the systolic pressure surge and dampen the force of the pulse as the flexibility of the arterial wall suppresses the pressure, thus their walls are thick and elastic. Blood pressure drops in the small arteries, and in the arterioles, blood flow is almost laminar.

Aorta

Aorta is the largest artery and it carries blood flow to the systemic circulation (Figure 3A). It starts from the aortic valve and ascending aorta. The first branches are the left and right coronary arteries, supplying the heart muscle. From the aortic arch the branches supply the arterial circulation of the neck, head and upper extremities: right the brachiocephalic trunk from which the right common carotid artery and the right subclavian artery originate and left the common carotid artery and the left subclavian artery which originate in the aortic arch. The descending aorta and the abdominal aorta branch out to supply the bronchus and the esophagus and the thoracic cavity, and the organs in the abdomen and the pelvic area, respectively. Aorta ends in a bifurcation which branches into iliac arteries for the lower limbs. Walls of the aorta are thick and elastic to compensate the pressure surge of the heart.

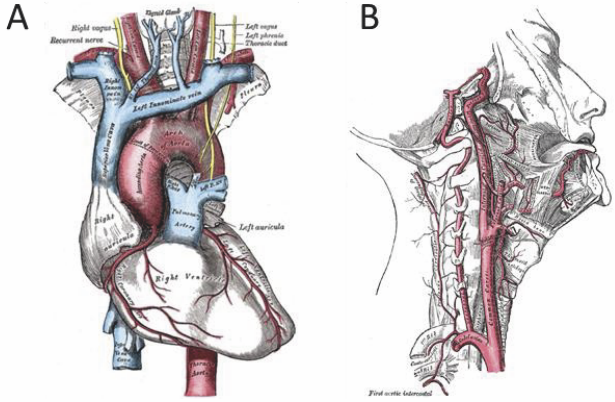

Figure 3. Thoracic aorta and its branches, and the right carotid artery A. Aorta is the largest artery and it begins after an aortic valve from the left atrium of heart. Coronary arteries start from cusps of the aortic valve and they carry blood to a heart muscle. Carotid arteries (B) branch from the aorta and carry blood to head. (Gray, 1918), plate 505, plate 513. (PD-Art) Public Domain

Coronary arteries

Coronary arteries aorta (Figure 3A) emerge, from inside of cusps of the aortic valve (Figure 1) called sinuses of Valsalva, and they supply oxygen and nutritients to the heart muscle. (Villa et al., 2016). The largest coronary artery is the short left main coronary artery. (Olivier et al., 2012). The left main coronary artery and its branches (left anterior descending artery and left circumflex artery (LCx) supply blood to the left ventricle and the left atrium, while the right coronary artery (RCA) branches supply blood to the right ventricle and atrium. Posterior descending artery and posterolateral artery are branches of either the RCA or LCx, and thus determine the dominance of the coronary artery system (Kini, Bis, and Weaver, 2007). In the right dominance system RCA sypplies the inferior segment of the left ventricle as well as the inferoseptal segment of the left ventricle, and this is more common (80—85 % of the cases) than the left dominance, in which LCx is the supplying vessel (Kini, Bis, and Weaver, 2007). The main branches of the coronary arteries may also be connected to each other and form collaterals that can provide blood supply to the the heart muscle in case of occlusion of the other branch (Werner, 2014). The lumen diameters of coronary arteries close to the aortic valve are the largest and become narrower branch by branch. (Dodge et al., 1992).

Carotid arteries

Common carotid arteries (Figure 3B) are large branches of aorta on the left and brachiocephalic artery on the right, on either side of the neck. They carry blood to the head and cranium. Common carotid artery (CCA) bifurcates into internal carotid artery (ICA) and external carotid artery (ECA). ECA branches to provide circulation to extra-cranial part of head. ICA supplies blood flow to intracranial arteries. It branches into several sections, which include ophthalmic artery and an artery ring that branch into cerebral arteries (Circle of Willis).

Structure of the arteries

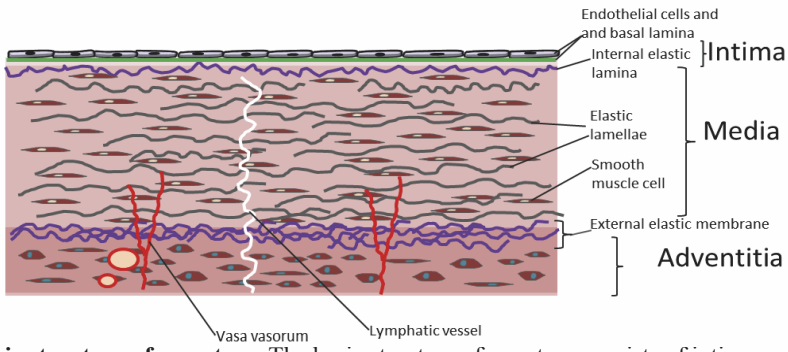

Figure 4 Basic structure of an artery. The basic structure of an artery consists of intima, media and adventitia. The intima consists of the endothelium and internal elastic lamina. A dense internal elastic lamina separates intima from media, and only small particles such as high density cholesterol can cross it. Media is nourished by vasa vasorum that originate from the adventitia and drained by lymphatic vessels, that also come from the adventitia.

The basic structure is similar in all arteries (Figure 4). In every artery there is an endothelial cell layer where the endothelial cells line the lumen to contain the blood flow inside of the artery. Under the endothelial cells and a basal lamina, there is loose connective tissue. An elastic layer, internal elastic lamina, is the border between intima and media. Media consists of smooth muscle cells and fenestrated elastic plates or laminae.

Between adventitia and media, there is an external elastic lamina. (Figure 4) External elastic lamina separates the media from adventitia, which consists of loose connective tissue.

In small arteries, where the hemodynamic forces and wall tension are low, the intima can be very thin, consisting only of the endothelial cells and the internal elastic lamina (Stary et al., 1992). In the large arteries, however, where the dampening of the pressure surges are necessary, the layer between the endothelial basal lamina and internal elastic lamina thickens into cushioning and energy-compensating proteoglycan-rich intima (Stary et al., 1992). The luminal side of normal intima is rich in proteoglycans, a thick net-like mesh. The proteoglycans, containing a core protein to which glycosaminoglycans such as dermatan sulfate, chondroitin sulfate and hyaluronic acid are attached to, are secreted by intimal smooth muscle cells (Wight and Ross, 1975a; Wight and Ross, 1975b). Internal elastic lamina is a dense mesh-like mat of elastic fibers, in which there are small holes called fenestrations (Kwon et al., 1998). It isolates the intima from the media, so that only smallest particles like high density lipoprotein (HDL) and intimal fluid are able to pass through (Kwon et al., 1998; Smith, 1990). Some areas, however, like bifurcation curvatures and branches, lack a well-defined internal elastic lamina (Stary et al., 1992). No blood vessels or lymphatic vessels are found in a healthy intima. The cells are only fed with nutrients and oxygen by diffusion from the blood stream in the lumen.

Outwards of the internal elastic lamina, there is a layer of smooth muscle cells, tunica media. In the small arteries it can be only one layer of smooth muscle cells while in the large arteries it consists of several layers of smooth muscle cells and elastic laminae. Medial smooth muscle cell layer provides arteries their ability to stretch and contract and thus maintain the arterial tonus. The smooth muscle cells are circumferentially arranged to surround the lumen, and between them there are elastin fibers. On the outer border of media external elastic lamina separates media from adventitia. Adventitia (300-500 µm thick, (Waller et al., 1992)) consists of elastic fibers and unorganized connective tissue. It serves to attach the arteries into tissues while it also contains specific blood vessels, nerves and lymphatic vessels to nourish and drain the arteries themselves. The lymphatic vessels and *vasa vasorum* reach only the media (Michael Munro and Path, 2012).

Lipoproteins

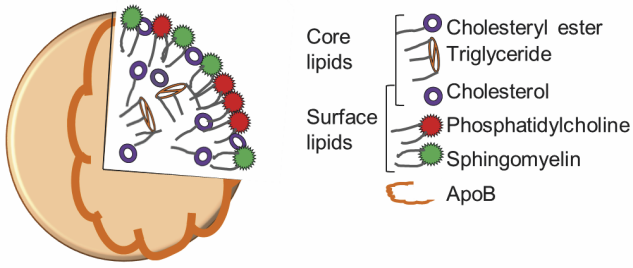

Figure 5. A schematic picture of the structure of an apoB--containing lipoprotein.

Lipoprotein particles transport nonpolar lipids in the blood stream. The lipoprotein particles consist of a protein moiety, a single layer phospholipid surface and a core containing nonpolar lipids (Figure 5). Next, lipoproteins will be discussed.

Proteins in chylomicrons, VLDL, IDL, LDL, and HDL

ApoB-48 and apoB-100 are structural proteins with which the lipids are incorporated in the particle. There is only one ApoB-48 or apoB-100 in a particle. They are integral proteins, which only gain their proper conformation when bound to lipid (Fisher, Lake, and McLeod, 2014). ApoB-48 found in chylomicrons is an intestinal splice variant product of apoB-100 gene, containing the N-terminal 48 % of the apoB-100 protein, where the name is derived from (Chen et al., 1987). ApoB-100 is the integral protein of VLDL, IDL, and LDL. ApoB-100 contains 4536 amino acids, the calculated mass of its unglycosylated form is 512 000 Da, making it one of the largest monomeric proteins (Knott et al., 1986; Law et al., 1986; Scott et al., 1987). When fully glycosylated, the protein's molecular weight is 550 000 (Cardin et al., 1984; Swaminathan and Aladjem, 1976). Its has approximately 40 % α -helix, 20 % β-sheet, and 20 % random structures (Chen et al., 1987; Law et al., 1986; Scott et al., 1987). The α -helices of apoB-100 contain hydrophobic and charged amino acid residues and are thus well adapted to bind lipid surfaces (Das and Gursky, 2015).

Nascent apoB-100 protein is lipidated as it is translated into mature protein in the endoplasmic reticulum (Fisher and Ginsberg, 2002; Olofsson and Boren, 2012; Rustaeus et al., 1998), through several lipid binding sites in the protein structure (Scott et al., 1987; Zhou, Fisher, and Ginsberg, 1998). Lipids are included in a quality control of the forming lipoprotein particle: polyunsaturated fatty acids are easily peroxidized. As they are incorporated in the nascent lipoprotein, the particles contain lipid peroxides, which can lead to aggregation of the incorrectly lipidated apoB (Pan et al., 2008). Such particles are substrates to post-ER presecretory proteolysis, and are therefore not released into circulation but degraged intracellularly (Pan et al., 2004; Pan et al., 2008).

Along with the apoBs, the lipoproteins can have exchangeable lipoproteins carried with them. Several copies of each exchangeable apolipoproteins can be found in the larger lipoprotein particles, chylomicrons, VLDL, and IDL. These particles carry apoE, apoC-III and II, apoA-I and II, and apoD, all of which they gain mainly from circulating HDL-particles (Havel, Kane, and Kashyap, 1973; Nakajima et al., 2011). In the LDL-particles, there are only trace amounts of apolipoproteins other than apoB-100 (Bancells et al., 2010; De Castellarnau et al., 2000). LDL can also carry some acute phase proteins like serum amyloid A (Bancells et al., 2010). HDL has two pieces of scaffolding proteins, usually two to three copies of apoA-I (Huang et al., 2011). Every fourth particle, however, has heterodimers of apoA-I and apoA-II. The HDL-particles can contain also other exchangeable apolipoproteins. ApoE is a largely α-helical protein (Chen, Li, and Wang, 2011) synthesized mainly in the liver (Wu and Windmueller, 1979; Zannis et al., 1981). It has affinity to LDL-receptor and proteoglycans (Ji, Pitas, and Mahley, 1998). Also macrophages (Zhang, Gaynor, and Kruth, 1996), especially foam cells (Laffitte et al., 2001) express and secrete apoE, and to a smaller extent also adipocytes and cells of small intestine (Laffitte et al., 2001). It is transferred to apoB-containing lipoproteins in circulation from HDL-particles. ApoC-III is another component of VLDL, also a small exchangeable lipoprotein that also is found in HDL. Liver cells express it, and it consists of 79 amino acids and contains several amphipathic α-helices (Gangabadage et al., 2008). All exchangeable apolipoproteins have an amphipathic α -helical structure that can easily associate to lipid surfaces, and can also transiently dissociate from the lipoprotein particle surfaces (Das and Gursky, 2015).

Chylomicrons, VLDL, IDL, LDL, HDL

In the normal cholesterol metabolism there are two pathways (Brown, Kovanen, and Goldstein, 1981). The first is an exogenous pathway, which includes the cholesterol derived from dietary sources and then transported eventually to liver in the chylomicros and chylomicron remnants (Figure 6). The second pathway

is the endogenous pathway (Figure 6), where the liver produces TAGs out of carbohydrates and fatty acids and produces the apoB-100-containing lipoproteins in which the TAGs along with cholesterol, as CE, are transported to extrahepatic cells. This endogenous pathway can be devided into two branches: VLDL, intermediate density lipoproteins (IDL), and LDL transport cholesterol to the extrahepatic tissues, and in reverse cholesterol transport, when HDL transports cholesterol from the peripheral cells to the liver. (Figure 6) Next, these lipoproteins will be discussed in more detail.

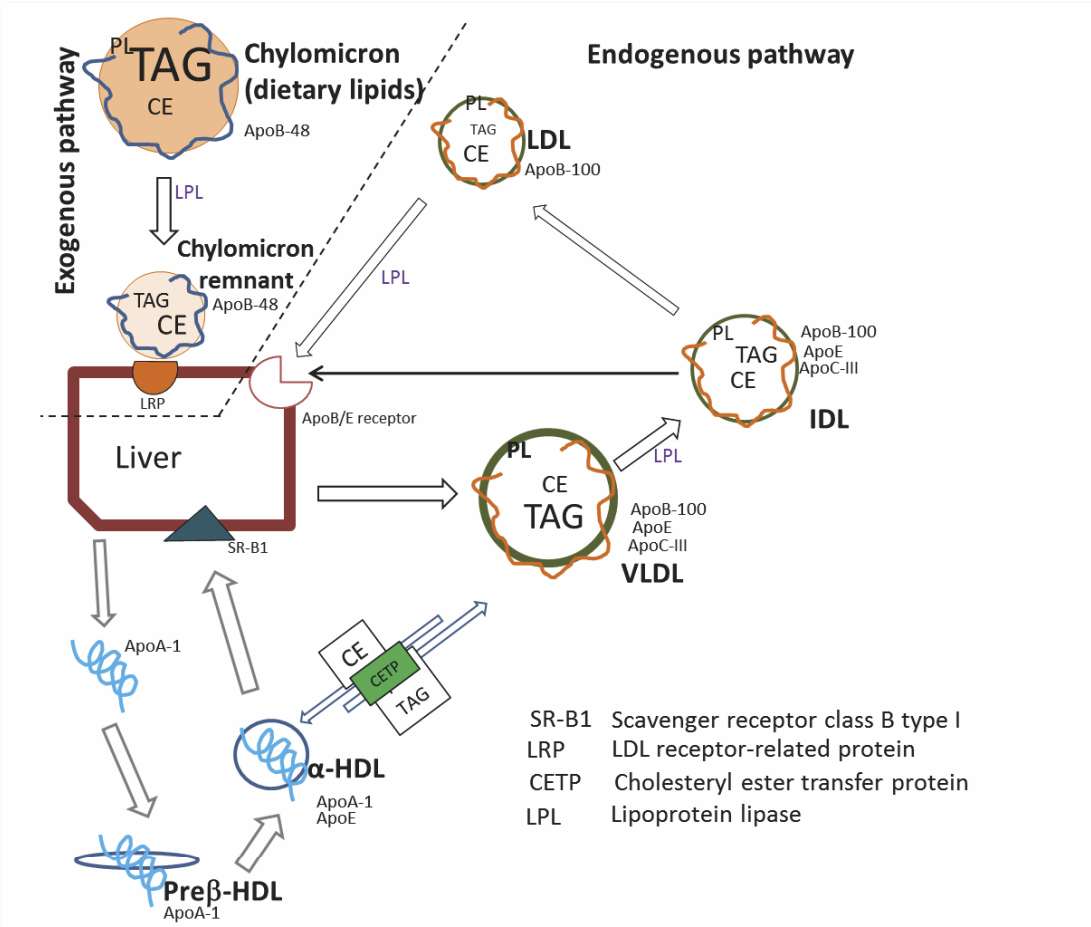

Figure 6. Schematic picture of lipoprotein metabolism.

Chylomicrons

Lipoproteins carry hydrophobic lipids and lipid soluble substances, such as α-tocopherol, α- and β-carotene in the blood stream. The first in line are chylomicrons that form in the small intestine out of the dietary lipids. The ingested lipids have been disassembled into 2-monoacyl glycerol and fatty acids by pancreatic lipase, absorbed by intestinal cells, and in the cells, reassembled into trigacylglycerols (TAG). Chylomicrons, as other lipoproteins as well, consist of amphipathic, phospholipid-rich and unesterified cholesterol-rich surface and hydrophobic core. In the chylomicron core, there are TAG, cholesteryl esters

(CE), and also small amounts of unesterified cholesterol. In the capillaries, lipoprotein lipase (LPL) lipolyzes the TAGs from the chylomicron particles to be used for energy in the peripheral tissues (Redgrave and Zech, 1987) or, to much greater extent, to be stored in the adipose tissue. Resulting chylomicron remnants, which have been stripped of most of their TAGs, are promptly taken to be processed in the liver, where remaining TAGs, cholesterol (esterified and unesterified), and phospholipids as well as the amino acids of the protein moiety are repurposed asVLDL (Grundy, 1978) (Figure 6).

VLDL, IDL, and LDL

VLDL-particles are loaded in the liver with TAGs and CEs. TAGs are the major lipid group (64 %) in VLDL, while proportion of chylesteryl esters is much lower (Table IB). The surface of VLDL-particle consists of phosphatidylcholines (PC) and sphingomyelins (SM), but some unesterified cholesterol is also buried in between the surface phospholipids. As VLDL releases most of its TAGs by the action of lipoprotein lipase, it gains density and becomes first IDL, and finally LDL (Table IA) (Figure 6).

LDL lipidome is dominated by CEs (nearly 50 % of total lipids of LDL,Table IB), which it distributes into cells for construction of hormones, bile acids, steroid hormones, vitamin D, and as a structural lipid of cell membranes. In the core of LDL, there are also unesterified cholesterol, TAGs, and fatty acids. Unesterified cholesterol is found also on the surface of the LDL-particle, and the surface contains as much as two thirds of the total unesterified found in the particle (Hevonoja et al., 2000).The surface of the LDL-particle consists of mainly of phospholipids, of which the PC are the most abundant, and SMs are the second most abundant phospholipid group. Also minor quantities of phosphatidylethanolamine and phosphatidylinositol are found. One LDL-particle consists of 25 % protein and 75 % of lipid (Goldstein and Brown, 1977).

The physical and chemical characterist of VLDL and LDL

VLDL is 30 to 80 nm in size and its density has been determined to be 0.95-1.006 g/ml (Methods in enzymology, vol 263 (Chapman, 1986). Once the lipoprotein has reached the density 1.019-1.063 g/ml and the size 18-30 nm, it is called LDL.

Ratio of cholesterol to total phospholipid (Chol/PL) can be used to describe the ratio of surface phospholipids to the core lipids in the circulating lipoproteins and it is often calculated when the degree of lipolysis of the lipoproteins is analyzed. Thus it is necessary to discuss the values of the normal circulating lipoproteins. In VLDL, the ratio of Chol/PL is 0.8 (Winocour et al., 1992) (Table IB). According to Chao and co-workers, in LDL the ratio Chol/PL is 2.5. (Chao et al., 1992), showing an increase in the proportion of cholesterol when compared to VLDL. Havel and co-workers measured the Chol/PL-ratio as they fractioned human plasma lipoproteins with ultracentrifugation (Havel, Eder, and Bragdon, 1955). In the density fraction $d < 1.019$, which contains VLDL and IDL, the ratio was 0.82, and in the density fration $d = 1.019–1.063$ containing LDL, the ratio was 1.39 (Havel, Eder, and Bragdon, 1955). Another ratio, PC to SM (PC/SM) can be utilized when the lipoproteins are analyzed. Holcapek and co-workers measured phospholipid characteristics of plasma lipoproteins and according to their study, PC/SM-ratio was 3.6 in VLDL and 2.1 in LDL, measured with ultrahigh - performance liquid chromatograph - atmospheric pressure chemical ionization - mass spectrometry (Holcapek et al., 2015), and earlier Esterbauer and co-workers, and Sommer and co-workers have reported similar results (2.4, 2.4, and 2.3, respectively) (Esterbauer et al., 1992; Sommer et al., 1992).

HDL

HDL is generatedeither by the action of lipoprotein lipase on VLDL which generates HDL particles when the excess phospholipid surface is dissociated from VLDL-particles, or as the nascent apoA-I collects lipids

from peripheral cells. Lipid-poor HDL is discoidal in shape and only gains spherical form after it takes up unesterified cholesterol and phospholipids from peripheral cells by a process mediated mainly by the transmembrane cholesterol transporter ATP-binding cassette A1 (ABCA1) (Alexander et al., 2011; Lee-Rueckert, Escola-Gil, and Kovanen, 2016) (Figure 6). The unesterified cholesterol in the discoidal HDL is esterified by the lecithin-cholesterol acyltransferase (LCAT), and the formed CEs then gradually form a core, so leading to the generation of mature spherical HDL particles. The CEs of the mature HDL particles can then be transferred from the HDL particles, catalyzed by the CE transfer protein (CETP) in exchange for TAGs, to VLDL and IDL particles (Barter et al., 2003; Barter, Hopkins, and Calvert, 1982). Ultimately the CEs are taken up by hepatocytes either directly from the mature HDL particles via the scavenger receptor class B type I (SR-BI) receptor, or via the hepatic LDL receptors when the apoB-100-containing lipoproteins (mainly LDL particles, or VLDL and IDL to a smaller extent) are taken up by these receptors (Lee-Rueckert, Escola-Gil, and Kovanen, 2016).

Nearly half of the lipidome of mature HDL isolated from serum consist of phospholipids, however, the composition may differ between different HDL subclasses (HDL_2 and HDL_3) (Wiesner et al., 2009). In nascent HDL most of the phospholipids are PC, and PC/SM ratio can be even 7 (Duong et al., 2006). In mature HDL the phospholipid contents vary between HDL classes (Kontush, Lhomme, and Chapman, 2013). Phospholipid content makes HDL is the major carrier of phospholipids in circulation (Wiesner et al., 2009), and up to 15 % of phospholipids can be LPC (Kontush, Lhomme, and Chapman, 2013). Most of the CEs in HDL is cholesteryl linoleate, and that is enriched especially in HDL_3 (Kontush et al., 2007).

Table IA. Lipoprotein characteristics. Apolipoproteins, protein- and lipid content, density, and particle size of plasma lipoproteins.

	Structural protein	Exchangeable apolipoproteins	Proteins (% dry weight)	Total Lipid (w %)	Density (g/ml)	particle size (nm)
Chylomicron	apoB-48	A-I,A-II, A-IV, C-I, C-II, C-III, E	1—2	99	<0.95	75—1200
VLDL	apoB-100	C-I, C-II, C-III, E	5—10	91	0.95—1.006	30—80
IDL	apoB-100	C-I, C-II, C-III, E	15—20	91-80	1.006—1.019	25—35
LDL	apoB-100	Trace amounts of E	18—25	75	1.019—1.063	18—30
HDL	apoA-I, A-II	A-I, A-II, A-IV, C-I, C-II, C-III, E	40—55	44	1.063—1.210	5—12
HDL_2	apoA-I, apoA-II	A-I (65%), A-II (10%), C (10-15%), E (3-5%)	35—40	59	1.063—1.125	8—11
HDL_3	apoA-I, apoA-II	A-I (62%), A-II (23%), A-IV (trace), C (5%), E (1%)	54—59	40—45	1.125—1.21	6—9

(Hilbert T, 2007), (Chapman, 1986), (Pownall H.J., 1999), (Esterbauer et al., 1992)

Table 1B. Lipoprotein characteristics. Lipid compositions of plasma lipoproteins.

	Total Phospholipids	Phosphatidyl - cholines	Lyso PC	Sphingo myelins	Neutral lipids	Triacyl– glycerols	Cholesteryl esters	Unesterified cholesterol
Chylomicrons (%)*	7					86	3	2
VLDL (%)*, £	18	14	0.8	2	84	55	12	7
IDL (%)*	19	15		4	81	23	29	9
LDL (%) $, ***	27.5	13.8	2.4	5.6	77	5.2	48.9	18.3
LDL (moles/mole LDL)$	900	450	80	185	2370	170	1600	600
HDL (%)******	33	33-45	0.5-5	5-10	35-52	5-12	30-40	5-10

* (Pownall H.J., 1999), **(Hussain, 2000), $ (Hevonoja et al., 2000), ***(Esterbauer et al., 1991), ****(Kontush, Lhomme, and Chapman, 2013; Wiesner et al., 2009), £ (Chapman, 1986)

Main lipids in the lipoproteins

*Cholesterol and cholesteryl est**er***

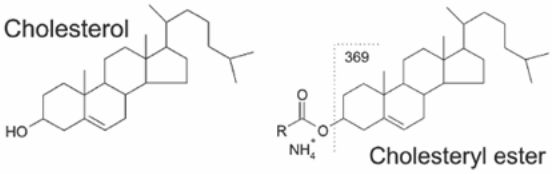

Figure 7. Cholesterol and cholesteryl ester. adapted by (Liebisch et al., 2006)

Cholesterol (Figure 7) ($C_{27}H_{45}OH$) has tetracyclic ring structure of sterols, which makes the structure very rigid. The hydroxyl (-OH) group in C3 is important for polarity and hydrogen bonding, and the aliphatic side chain in C17 increases hydrophobicity. It is a lipophilic, nearly water-insoluble (1.8 mg/l), waxy substance that crystallizes easily. Cholesterol crystallizes with triclinic basic structure, where crystals have no 90 degree angles (Shieh, 1977).

Cholesterol is transported and stored in esterified form, as CEs. CEs are formed when a fatty acyl chain is esterified with hydroxyl group in C3 (Figure 7). Esterified cholesterol is more hydrophobic than unesterified cholesterol, due to an aliphatic carbon chain of fatty acid. The length of the fatty acid determines the degree of hydrophobicity. Due to high hydrophobicity, CEs are transported in the core of lipoprotein particles.

Cholesterol biosynthesis pathway is a highly conservative multi-step pathway, in which one enzyme, hydroxyl-methyl glutaryl-CoA reductase controls the rate limiting step. This step can be inhibited with statins, and thus the synthesis of cholesterol is prevented.

Triacylglycerols

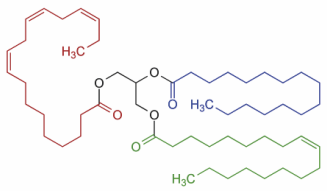

Figure 8. Triacylglycerol. In figure is depicted a 1-linolenic-2-palmitic-3-oleic triacylglycerol (TAG 52:4).

TAGs (Figure 8) are storage lipids, stored mainly in adipocytes, and can be hydrolyzed and used as energy in muscle, especially in heart muscle. TAGs are comprised of a glycerol backbone and three fatty acid chains, where fatty acids form ester links with the hydroxyl groups of glycerol. As neutral lipids the TAGs are highly hydrophobic. TAGs are the major form of fat in human diets. The ingested TAGs are dismantled into mono- and diglycerides and fatty acids in the intestinal lumen by pancreatic lipase, then taken up to the cells cells lining the intestine and reassembled back to TAGs for transport. TAGs are transported initially in the chylomicrons to the liver, and in the liver they are packed into very low density lipoprotein (VLDL) to be transported to the peripheral tissues.

Phospholipids

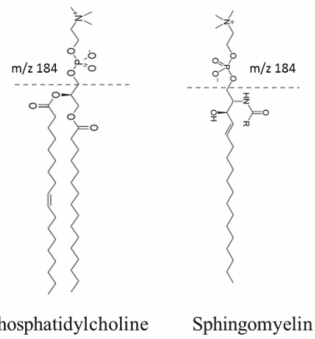

Figure 9. Phosphatidylcholine and sphingomyelin. Letter R in sphingomyelin stands for an acyl group.

Phospholipids are a heterogeneous group of amphipathic lipids, and they compose 23 % of the total lipids of low density lipoprotein (Esterbauer et al., 1992). The major groups of phospholipids are glycerophospholipids, which consists of the same glycerol backbone as TAGs, two fatty acyl side chains and an amphipathic head group attached to the glycerol backbone via phosphate group. The main glycerophospholipid groups are PC (Figure 9), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS). In natural phospholipids, unsaturated fatty acids are often in the *sn*-2 position of the glycerol backbone, while the *sn*-1 position is often occupied by a saturated acyl chain (Beermann et al., 2005; Mitchell, Straume, and Litman, 1992). The degree of unsaturation affects the membrane rigidity (Mitchell, Straume, and Litman, 1992). Glycerophospholipid can be degraded to lysophospholipids. The glycerophospholipids lose an acyl group and as a result contain the amphipathic head group and one acyl chain attached to the glycerol back bone. PC can be hydrolyzed to lysophosphatidylcholine (LPC), where the acyl chain in *sn*-2-position is hydrolyzed as a result of enzymatic hydrolysis or oxidation. The responsible enzyme, PLA_2 and the hydrolysis will be discussed later in the chapter Phospholipase A_2, page 23

SMs (Figure 9) have a close resemblance of glycerophospholipids, but instead of *sn*-1 acyl chain they contain an aminoalcohol called sphingasine. The head group of glycerophospholipids can be either choline, serine, ethanolamine, inositol, or glycerol, and, similarly, in SMs a variety of head groups can be attached to phosphate group.

Fatty acids

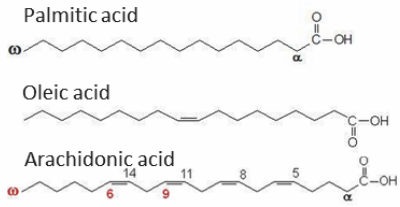

Figure 10. Fatty acids

Fatty acids are the building blocks of CEs, TAGs, and phospholipids. Fatty acids are aliphatic, unbranched hydrocarbon chains of 4—32 carbons, usually with an even number of carbons, a carboxyl group in the end. They can be saturated with no double bonds or unsaturated with varying amounts of double bonds (Figure 10). The most relevant fatty acids in human physiology are palmitic acid (16 carbons, no double bonds (16:0)), palmitoleic acid (16 carbons, one double bond (16:1)), myristic acid (14:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), a-linolenic acid (18:3), arachidonic acid (20:4), eicosapentaenoic acid (20:5), and docosahexaenoic acid (22:6). Fatty acids can also be found free in lipoproteins, for example low density lipoprotein (LDL) contains less than 1 % free fatty acids (Esterbauer et al., 1992).

Aortic stenosis and atherosclerosis

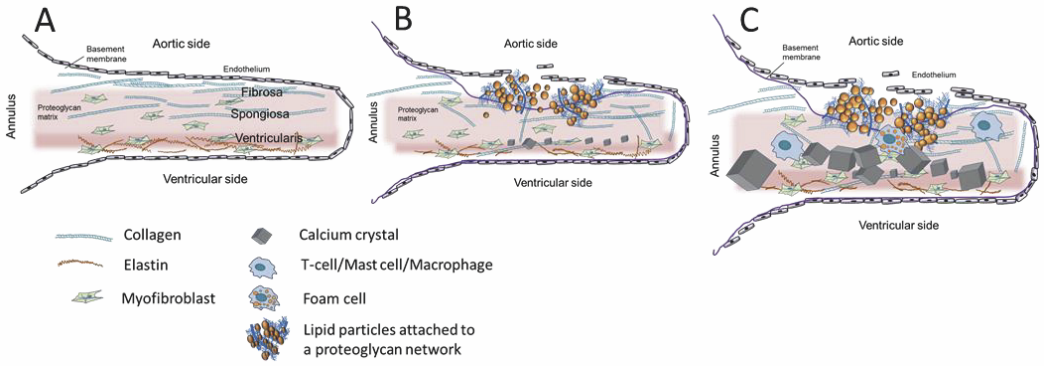

Figure 11. A Normal aortic valve leaflet. B. Aortic sclerosis. Lipids accumulate in the proteoglycan matrix and microscopic calcium crystals begin to form in the leaflet. C. Aortic stenosis. Lipid droplets have accumulated in the proteoglycan-containing matrix and calcification is extensive. The leaflets also contain inflammatory cells and lipid filled foam cells.

The onsets of aortic stenosis and atherosclerosis are gradual, and the changes in the tissues are stealthy. Atherosclerosis is an intimal disease, initiated by lipoproteins entering the arterial intima and retaining in the dense proteoglycan matrix (Williams and Tabas, 1995). In the course of the disease, the healthy thin intima thickens to contain retained lipoproteins and inflammatory cells such as macrophages, which will become foam cells after consuming the modified retained lipoproteins. Aortic valve is a distinctive part of vascular system, and aortic stenosis has many similarities to atherosclerosis (Otto, 2004), such as increasing of the proteoglycan matrix and lipid accumulation. In early stage of aortic stenosis (aortic sclerosis) the aortic leaflets are starting to fill with lipid and contain microscopic calcific nodules (Figure 11), (Freeman and Otto, 2005). Aortic stenosis is characterized not only by lipid accumulation, but also by extensive calcified nodules in the valve leaflets (O'Brien et al., 1996).

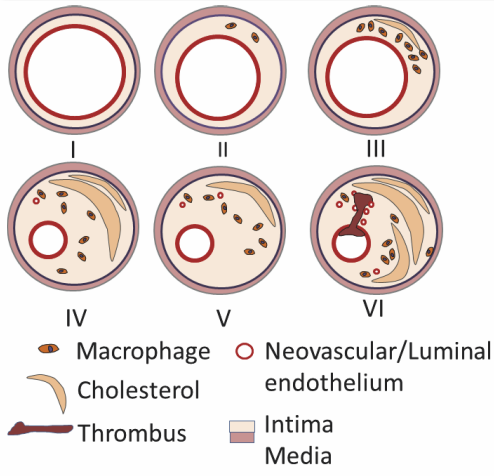

Figure 12. Schematic picture of normal intima (I), Stages II, III, IV, V, and VI. In stage II, macrophages have started to accumulate in the thickened intima, in Stage III early lipid accumulation can be found. In stage IV, lipid core has begun to form near the internal elastic lamina and the intima has thickened remarkabaly. Stage V is a fibrous lesion, containing thick layers of connective tissue and a thick fibrous cap. In stage VI, hematoma and thrombus are found. Modified from (Stary et al., 1995)

Lipids accumulate in the arterial intima as plasma lipoproteins enter the intima and are retained in the proteoglycan matrix and macrophage-derived foam cells fill up with lipid droplets. Stary et al have defined the different stages of atherosclerosis from normal arterial intima to very advanced lesions (Figure 12), and describe the distribution of the lipids with photomicrographs and electron microscopy images on each stage (Stary, 1994; Stary et al., 1992; Stary et al., 1995; Stary et al., 1994b).

Proteoglycans in the valve leaflets and in arterial intima

In normal aortic valve leaflets, versican, decorin, hyaluronan, and biglycan are the dominant proteoglycans. Myofibroblasts in fibrosa secrete especially decorin, but the total proteoglycan content of fibrosa is lower than in the other layers (Stephens, Chu, and Grande-Allen, 2008). Proteoglycan distribution of the spongiosa differs from fibrosa and ventricularis as the spongiosa contains more versican and hyaluronan than the other two layers do (Stephens, Chu, and Grande-Allen, 2008). Biglycan is overexpressed in stenotic aortic valves (O'Brien 2004, Song 2012), and it is found in the fibrosa and ventricularis layers (Djerbali 2010).

In electron microscopy images of arteries, collagen bundles are frequently found (Nievelstein et al., 1991; Simionescu et al., 1986), and collagen was found to co-localize with proteoglycans (Wight and Ross, 1975a). In arterial intima, smooth muscle cells secrete collagen, which in part stabilizes the forming neointima, but also binds proteoglycans, such as biglycan (Schönherr et al., 1995b) and decorin (Schönherr et al., 1995a),

and in immunohistochemistry is found to co-localize with them in the normal and atherosclerotic arteries(Riessen et al., 1994). In ta study of arteries of non-human primates (*Macaca nemestrina*), normal arteries contained mainly perlecan, decorin, and biglycan in the vicinity of endothelial cells, and perlecan, decorin, biglycan, and versican where smooth muscle cells were found (Evanko et al., 1998). In fibrous plaques, they found that different combinations of proteoglycans were formed in different areas of the plaque: smooth muscle cell –rich areas contained perlecan, biglycan, versican, and hyaluronen, whereas areas rich in macrophages were rich in decorin, but poor in versican, and the fibrous cap was shown to be poor in perlecan and decorin, but rich in versican and biglycan (Evanko et al., 1998; Wight and Ross, 1975a; Wight and Ross, 1975b).

Matrix remodeling of aortic valve leaflets and intimal thickening in arteries

In aortic valves, the early lesion stage manifest as thickening (Otto et al., 1994). Cell density is increased especially in the fibrosa, while in the spongiosa layer, the amount of collagen is increased (Olsson 1994b). Aortic valve sclerosis is often considered as an early stage of aortic stenosis, as the changes that are at their strongest manifested in the aortic stenosis, can be found already in the aortic valve sclerosis. Such changes are accumulation of foam cells, fibroblasts expressing smooth muscle cell-like features, and early mineralization (Prasad and Bhalodkar, 2004).

Diffuse intimal thickening in arteries is likely to occur at long non-branching segments of arteries, or paradoxically, at sites around branches (Nakashima et al., 2002; Stary et al., 1992). This intimal thickening starts as an adaptive phenomenon. A prerequisite of the pathological intimal thickenings are the benign, physiological adaptive intimal thickening sites, which will eventually become pathological. Pathological intimal thickenings contain retained and modified lipoproteins in the increased proteoglycan matrix, inflammatory cells, including macrophage and smooth muscle cell -derived foam cells, and in the advanced stage, a necrotic core that has been formed by dead foam cells (Nakashima et al., 2007).

Nakashima studied arteries from fetuses to adults at their 30's (Nakashima et al., 2002). His group showed that diffuse intimal thickening was present in coronary arteries already in the prenatal stage, while in the distal arteries like in the splenic artery, only minimal adaptive intimal thickening could be detected in young Japanese adults, whose coronary arteries had already prominent adaptive intimal thickening (Nakashima et al., 2002). The exact point when an adaptive intimal thickening in the arteries turns pathological is hard to distinguish, as both adaptive and also the pathological intimal thickenings may coincide in atherosclerotic-prone areas (Riessen et al., 1994; Stary et al., 1992).

In areas where hemodynamic forces, wall tension, eddy currents and shear stress affect the artery (comprehensivlye reviewed by (Chiu and Chien, 2011) and cause an endothelial erosion where the endothelial cell layer is compromised and platelets attach to the surface (Mäyränpää et al., 2007). Endothelial dysfunction is associated with both aortic sclerosis and atherosclerosis (Anderson et al., 1995; Chenevard et al., 2006; Poggianti et al., 2003).

Lipoproteins in aortic valve and arterial wall

In work of Nievelstein et al (Nievelstein et al., 1991) a bolus of LDL particles was injected into a rabbit, and after just two hours, the lipid particles were found within the aortic proteoglycan matrix. Some of the particles seemed to be aggregated and even fused together to form larger particles. However, no such clusters or aggregates were present in the injected lipid prior to injection (Nievelstein et al., 1991). Nievelstein-Post and co-workers used also rabbit heart valves (aortic valve and atrio-ventricular valve) as a model tissue to examine how lipoproteins enter the intima and associate with the extracellular matrix (Nievelstein-Post et al., 1994). In this experiment, LDL entered the valve within two hours of incubation, and the authors concluded

that the accumulation is due to interactions between the lipoproteins and the matrix in the intima. Stender and Hjelms (Stender and Hjelms, 1984) had performed a similar experiment on human subjects, injecting radiolabeled cholesterol-containing lipoproteins into patients undergoing an operation to replace aortic valve. In the *in vivo* human experiment of Stender and Hjelms, a very small amount of radiolabeled lipoproteins entered the aortic intima 12 to 40 minutes after the injection. 24 hours after the injection of radio-labeled lipoproteins, the radioactive label accumulated in the aortic intima much more than in the media.

Frank and Fogelman show in an experiment where they feed rabbits with cholesterol-rich diet for 10 days, the aortic intima filled with lipid inclusions (Frank and Fogelman, 1989). They reported that no lipid is visible in the intima of the control rabbits, although, some granule-like structures were found attached in the fiber-like structures (Frank and Fogelman, 1989).

Animal experiments with genetically hyperlipidemic or cholesterol-fed rabbits show how intact endothelium is nearly impermeable to particles that are larger than LDL (Nordestgaard, Wootton, and Lewis, 1995; Nordestgaard and Zilversmit, 1988; Stender and Zilversmit, 1981), although previously Nordestgaard and co-workers had stated that lipoproteins that are smaller than 75 nm can enter the intima through the endothelium (Nordestgaard and Zilversmit, 1988). Endothelial damage will enhance the entrance (Frank and Fogelman, 1989). Plasma macromolecules, including apoB-containing lipoproteins have been shown to enter the intima through healthy endothelium through transcytosis (Simionescu, Gafencu, and Antohe, 2002). Accumulating apoB-containing lipoproteins can then lead to endothelial dysfunction and subsequent increase of endothelial layer permeability, as products of their lipolysis will damage the endothelium, and allow the particles to penetrate into the intima (Eiselein et al., 2007). In porcine and murine arteries, (Kwon et al., 1998) LDL was found to bind in the branching cites of the large arteries if the elastin basement membrane was compromised. Similar effect was found in porcine valves, where LDL bound to elastin-poor surface (Neufeld et al., 2014).

Nakashima and co-workers (Nakashima et al., 2007) demonstrated that accumulation of lipoproteins in the intimal matrix is the initial phenomenon in lesion development. In the intima, the first deposits are diffuse and not visible in light microscopy (Nakashima et al., 2007; Nievelstein-Post et al., 1994; Nievelstein et al., 1991). As internal elastic lamina, despite its fenestrations, inhibits spontaneous diffusion of lipoprotein particles and as arterial intima lacks lymphatic vasculature (Nakano et al., 2005), the lipoprotein particles are not drained from the intima, but rather accumulate there. Also aortic valve leaflets lack lymphatic vessels (Bradham et al., 1970), however no internal elastic lamina exists in the valve. In both atherosclerotic artery intima and in stenotic aortic valves lymphatic vasculature increases, still in both tissues, the imbalance between influx of lipoprotein particles and drainage through the lymphatic vasculature remains (Nakano et al., 2005; Syväranta et al., 2012). Due to the imbalance, lipoprotein particles accumulate in both arterial intima and aortic valve.

ApoB, apoE, and apoA-I are found to be able to interact and co-localize with biglycan in the atherosclerotic lesions (O'Brien et al., 1998), and all these apolipoproteins also co-localize with biglycan in stenotic aortic valve lesions (Lommi et al., 2011; O'Brien et al., 1996). Biglycan is shown to bind apolipoproteins B-100 and E (O'Brien et al., 1998; Olin et al., 2001) and apo A-I has been shown to perfectly co-localize with biglycan by immunohistochemical stainings, most likely as part of the apoE-containing VLDL, VLDL-remnants or HDL-particles (O'Brien et al., 1998; Olin et al., 2001). In immunohistochemistry, versican, the most abundant intimal proteoglycan (Camejo et al., 1998; Theocharis et al., 2001), has been found not to co-localize with apoB-100 or apoE both in human coronary arteries (O'Brien et al., 1998) or in mice aortas (Kunjathoor et al., 2002), although LDL has been shown to have high affinity on versican-like proteoglycans (Camejo et al., 1993). This may also be due to loss of apoB-100 immunoreactivity in atherosclerotic lesions (Kruth and Shekhonin, 1994).

Once the apoB-containing lipoproteins have entered the subendothelial space, they are retained in the proteoglycan matrix (Simionescu et al., 1986; Skålen et al., 2002). ApoB-100 has *in vitro* affinity towards proteoglycans via its basic amino acids, in particular near the LDL-receptor binding site (residues 3359-3369) (Skålen et al., 2002). ApoE-containing lipoproteins are able to attach the proteoglycans also via apoE's ionic affinity to biglycan, although the affinity of apoB to this proteoglycan is higher than that of apoE (Olin et al., 2001). In concordance with the Response-to-retention hypothesis, the retained lipoprotein particles in the proteoglycan matrix are susceptible to modifications by oxidation, proteolysis, and lipolysis (Williams and Tabas, 1995).

After the injection of LDL into the rabbit circulation, Nievelstein and co-workers observed aggregates of LDL-sized lipoproteins and also enlarged particles that they suggested to be fused lipoproteins (Nievelstein et al., 1991). Also Frank and Fogelman observed large clusters of lipid particles in the extracellular matrix of cholesterol-fed rabbits (Frank and Fogelman, 1989). Pentikäinen and co-workers showed in *in vitro* experiments that such aggregates can be results of agitation, immune-complexes, oxidation, proteolysis or lipolysis (Pentikäinen, Lehtonen, and Kovanen, 1996). Lipolysis, oxidation, and proteolysis will be discussed in more detail below, on pages 24, 26, and 27, respectively. *In vivo*, especially deeper in the intima, also acidic extracellular pH can contribute to aggregation of lipoproteins (Öörni et al., 2015). Intimal macrophages (Tabas, Williams, and Boren, 2007) and smooth muscle cells (Allahverdian, Pannu, and Francis, 2012) take up the aggregated lipoproteins and become foam cells. Foam cells will be discussed in a later chapter (Page 29).

Modifications of apoB-100-containing lipoproteins in atherosclerosis

Lipolysis and lipolytic enzymes

Lipoproteins from chylomicrons to LDL are targets of lipolysis. Via lipolysis, the TAGs in the TAG-rich lipoproteins are hydrolyzed to glycerol and fatty acids to be used for energy in the muscles and, on the other end, the excess fatty acids that were not necessary for energy will be stored in adipose cells or used as raw material for biosynthesis. CEs are hydrolyzed into cholesterol and fatty acids, and the unesterified cholesterol is used in peripheral steroidogenic cells to produce glucocorticoid hormones or steroid hormones. Phospholipids are hydrolyzed for production of signal transduction molecules, such as ceramides, fatty acids, eicosanoids, or inositol phosphates. These physiological lipolysis pathways are also found to affect pathological events in atherosclerosis. Next, the lipolytic enzymes will be examined, as they can be factors in atherogenesis by lipolyzing lipoproteins and have also effects on the smooth muscle cells or macrophages.

Sphingomyelinase

Ceramides are detected in atherosclerotic plaques and they have been linked to progression of atherosclerosis (Edsfeldt et al., 2016). Fitting to that, acid sphingomyelinase (SMase) is detected in the intima (Edsfeldt et al., 2016). It promotes lipoprotein retention within early atheromata and accelerates lesion progression (Devlin et al., 2008). Acid SMase (SM phosphodiesterase (EC 3.1.4.12) (Barnholz, Roitman, and Gatt, 1966), SMase) is secreted by both endothelial cells and intimal macrophages and it binds to matrix proteoglycan components and several types of collagen (Marathe et al., 1999; Marathe et al., 1998), and it has been found to have also mitogenic effect on smooth muscle cells in vitro (Auge et al., 1996). Secretion of SMase from the endothelial cells is stimulated by cytokines interleukin-1β (IL-1β), and interferons γ and β, as Marathe and co-workers showed in an in vitro experiment (Marathe et al., 1998). SMase hydrolyzes SM by removing the phosphocholine headgroup to produce ceramide that consists of sphingosine and a fatty acid. It is one of the enzymes that enhances lipoprotein aggregation and their affinity to human aortic proteoglycans (Öörni et al., 2005). Hydrolysis of LDL lipids by SMase distorts the protein conformation of apoB-100 bringing out hydrophobic parts of the protein (Sneck et al., 2012). This will induce aggregation of

the LDL-particles. Exhangeable apolipoprotein apoC-III in LDL increases the susceptibility of LDL to SMase (Schissel et al., 1998), by bridging SMase and membrane SM (Schissel et al., 1998) and this is notable particularly in LDL particles isolated from patients having type 2 diabetes (Hiukka et al., 2009). Hiukka and co-workers also show that LDL from patients with type 2 diabetes contain less ganglioside 1 (GM1) than non-diabetic LDL, and that low GM1-levels were associated with high apoC-III levels. Fanani and co-workers had previously shown that GM1 inhibits SMase (Fanani and Maggio, 1997), so Hiukka and co-workers postulated that low GM1 levels can lead to increased hydrolysis by SMase (Hiukka et al., 2009). The SMase-hydrolyzed LDL has also greater affinity to proteoglycans (Hiukka et al., 2009) and collagen (Marathe et al., 1999).

Phospholipase A_2

PLA_2-family enzymes catalyze the hydrolysis of a fatty acid that is esterified in the *sn*-2 carbon of the glycerol back bone in glycerophospholipids. Hydrolysis produces a free fatty acid and a lysophospholipid. Murakami (Murakami et al., 2015; Murakami and Taketomi, 2015) has very recently reviewed the secretory PLA_2-enzymes with great detail. There are ten major secretory PLA_2-species and each have favorite substrate specificity. The PLA_2s suggested to be significant in the development of atherosclerosis are PLA_2-IIa (Menschikowski, Hagelgans, and Siegert, 2006), PLA_2-III (Sato et al., 2008), PLA_2-V (Boström et al., 2007), and PLA_2-X (Bezzine et al., 2000) in the intima, and Lp-PLA_2 which travels with LDL particles (Kolodgie et al., 2006) and has activity towards oxidized LDL in vitro. Of these, PLA_2-IIa requires modified LDL as a substrate, but the activity towards native LDL is very modest (Hakala et al., 2001; Sartipy et al., 1999), whereas PLA_2-III, PLA_2-V and PLA_2-X are able to hydrolyze also native LDL phospholipids (Pruzanski et al., 2005; Rosengren et al., 2006; Sato et al., 2008). Apart from atherosclerosis, PLA_2-IIA -enzymes contribute to defence against pathogens, such as bacteria in the lung tissue (Movert et al., 2011). Group X PLA_2 is expressed in the gastrointestinal tract and takes part in the lipid digestion (Murakami et al., 2015) and PLA_2-III contributes to mast cell maturation (Murakami and Taketomi, 2015). Arachidonic acid, as source of eicosanoids, is one of the fatty acids released from the phospholipid. Expression of PLA_2-IIA or PLA_2-X in mammalian cells is integral in a release of arachidonic adic from cells, and they work in co-operation with cytosolic PLA_2-enzymes (Mounier et al., 2004). The release of arachidonic acid is enhanced by IL-1β (Mounier et al., 2004).

Hydrolysis of lipoproteins with PLA_2 has also been shown to affect their affinity to the proteoglycans. Lipolysis of LDL with PLA_2 increases remarkably the lipoprotein's affinity to proteoglycans and the same can be seen also in case of IDL- and VLDL-particles (Lähdesmäki et al., 2012). In native LDL, only one of the proteoglycan binding sites in apoB (site B, amino acid residues 3359-3369) is available (Boren et al., 1998b), but after lipolysis with PLA_2, also a second proteoglycan binding site (residues 3148-3158) is exposed and can bind to the proteoglycans, strengthening the binding (Flood et al., 2004). Unlike SMase, which is able to induce both fusion and aggregation of LDL-particles, PLA_2-lipolyzed LDL-particles were only aggregated (Öörni et al., 1998). Interestingly, in the presence of glycosaminoglycans like heparin, also PLA_2-treated LDL can form fused particles (Hakala et al., 1999).

Apart from the phospholipids, also neutral lipids get lipolyzed in the LDL particles, a phenomenon that, as described below, induces aggregation of the lipoprotein, enhances retention to the proteoglycans, and facilitates uptake by macrophages.

Lysosomal acid lipase

Lysosomal acid lipase (LAL) (belongs to a family of acid lipases EC 3.1.1.13)(Ameis et al., 1994; Hyun et al., 1969; Lohse, Chahrokh-Zadeh, and Seidel, 1997), also known as CE hydrolase, is a necessary enzyme in the metabolism of CE-enriched lipoproteins, as it hydrolyzes their CEs in lysosomes into unesterified

cholesterol and free fatty acids (Ameis et al., 1994; Goldstein et al., 1975). In addition to cholesteryl esterase activity, LAL also has triacylglyceridase activity on TAGs having medium length (or long) acyl chains (Ameis et al., 1994; Lohse, Chahrokh-Zadeh, and Seidel, 1997). In *in vitro* -experiments, LAL shows higher activity as TAG-hydrolase than as cholesteryl esterase (Ameis et al., 1994), but it is the only lysosomal lipase that hydrolyzes CEs within lysosomes (Dubland and Francis, 2015). Recently LAL has been found to regulate VLDL synthesis in mice (Radovic et al., 2016). In macrophages it is found in the lysosomal compartment and it catalyzes the deacylation of TAG and CE of lipoproteins that have been taken up by macrophages (Goldstein et al., 1975; Sando and Rosenbaum, 1985). The resulting unesterified cholesterol is then re-esterified into CE in the macrophage lipid droplets (Brown, Ho, and Goldstein, 1980; Goldstein et al., 1975), and the excess fatty acids are incorporated into phospholipids (Darmani, Harwood, and Jackson, 1993). (This process of macrophage CE uptake is described in detail in the chapter Foam cells, page 29). In addition to its role in lysosomes, LAL is also secreted into pericellular space upon macrophage activation (Hakala et al., 2003; Schissel et al., 1998), and as it shares sequences and active site structures with human gastric lipase, it is plausible that it is able to function outside of the lysosomes in the pericellular space, given that the necessary acid pH is achieved (Lohse, Chahrokh-Zadeh, and Seidel, 1997; Sheriff, Du, and Grabowski, 1995; Öörni et al., 2015). Haka and her group showed how macrophages can create an acidic environment in the extracellular space around them by secreting the lysosomal contents in the so called surface connected compartments, i.e. invaginations at the cell surface (Haka et al., 2009), and thus the CE hydrolysis can take place outside the macrophages. They also show that LDL-aggregates form these acidic compartments in a murine J774-macrophage model (Singh et al., 2016). Both Chao and co-workers (Chao et al., 1992) and Bhakdi and co-workers (Bhakdi et al., 1995) suggest, that for LAL to work, and to be able to properly reach the lipoprotein core CEs and hydrolyze them, LDL needs a preceeding modif oxidation or proteolysis. Only modest aggregation can be detected on cholesteryl esterase treated LDL (Öörni et al., 2000), while it forms then unesterified cholesterol -filled multilamellar bodies. Similar vesicles can be found also in aortic intima (Chao et al., 1992).

Lipoprotein lipase

Lipoprotein lipase (LPL) (EC 3.1.1.34) activity was first described by Hahn (Hahn, 1943) by its ability to clear lipids from the blood of dogs, however he did not yet recognize the enzyme itself, and Korn et al (Korn, 1955a) isolated this “clearing factor” from rat hearts. LPL can lipolyze TAGs into three free fatty acids and glycerol, using mainly tri-, di-, and monoacylglycerols as substrates (Korn, 1955b; Miller and Smith, 1973). It can also hydrolyze PC (Scow and Egelrud, 1976) and PE (Groot, Oerlemans, and Scheek, 1978) as substrates, but those are minor substrates compared to TAGs. The enzyme’s primary function is to hydrolyze the TAGs from circulating triacylglyerol-rich lipoproteins chylomicrons, VLDL, and IDL by binding as a dimer to endothelial cell proteoglycans in capillaries, and concomitantly to the lipoproteins, whose TAGs it lipolyzes. A necessary activator of LPL is apoC-II that chylomicrons and VLDL carry (Chung and Scanu, 1977). The resulting free fatty acids are to be used as energy by muscle cells or stored as TAGs in adipose tissue. Lipoprotein lipase is expressed also by tissue macrophages and it enhances VLDL, IDL, and LDL retention to proteoglycans mediated by LPL's binding to the phospholipid layer of the lipoprotein particles and thus bridging the two together (MacPhee et al., 1997; Pentikäinen et al., 2002). LPL is found to positively correlate with atherosclerosis in mice (Babaev et al., 1999; Van Eck et al., 2000), and in human macrophages LPL-gene is associated with foam cell formation as it increases VLDL and LDL uptake by macrophages (Olivier et al., 2012). Apolipoproteins apoC-I, C-II, and C-III, apoA-V, and apoE have been found to control LPL activity: apoA-V and apoC-II stimulates the TAG hydrolysis and apoC-I, apoC-III, and apoE inhibit it thus increasing circulating levels of TAG (Kersten, 2014). Also a group of angiopoietin-like (ANGPTL) protein family members can modulate the activity of LPL. ANGPTLs 3, 4 and 8, of which ANGPTL4 is a competitive inhibitor of LPL, reduce LPL activity and thus increase plasma TAG

concentration (Kersten, 2014). The role of ANGPTL 8 in mice has been found to increase plasma TAG by inhibiting LPL activity, and its actions have veen found to be tissue specific (Kersten, 2014), and the ANGPTL3 is supposed to work similarly as ANGPTL8. In a recent review by Tikka and Jauhiainen (Tikka and Jauhiainen, 2016) the role of ANGPTL3 role in lipoprotein metabolism is discussed in detail. Homozygous carriers of a rare loss of function mutation in the gene coding ANGPTL3 have a rare disorder familial combined hypobetalipoproteinemia, causing very low VLDL. LDL, and HDL levels (Tikka and Jauhiainen, 2016). Such loss of function mutations have been detected in some Italian, Spanish, and American families. The mutation carriers have been found not to suffer from serious adverse effects (Tikka and Jauhiainen, 2016).

Oxidation of lipoproteins

LDL oxidation enhances LDL aggregation in vitro, as shown by Pentikäinen and co-workers (Pentikäinen, Lehtonen, and Kovanen, 1996), and oxidized epitopes are found in arterial intimas (Ollikainen et al., 2016). The cholesterol moiety, the surface phospholipids, and the apoB-100 protein can all become modified via contact to transition metals, such as cupric ions (Cu^{2+}), as Nichols found already 1961 by dialyzing LDL against cupric ion solution (Nichols, 1961). ApoB-100 of LDL is susceptible to non-enzymatic proteolysis where it is degraded independent of proteolytic enzymes by endothelial cell-derived oxygen free radicals (Fong et al., 1987). Each lipid class produces distinct oxidation products: 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA) are derived from ω6 fatty acids by chain cleavage, and these can also modify proteins by covalently modifying the lysine residues of proteins, notably of the apoB-100 of LDL, and so generate 4-HNE-modified and MDA-modified LDL (Esterbauer et al., 1991; Fruebis, Parthasarathy, and Steinberg, 1992) The positively charged, "active" lysine residues that contribute to LDL binding to the proteoglycans are exposed on the surface of LDL particle and have lower pK compared to the other lysines of apoB-100 (Lund-Katz et al., 1988). The formation of 4-HNE-lysine and MDA-lysine adducts decreases the number of active lysine residues in apoB-100 of LDL and thus reduces the ability of LDL to attach to proteoglycans (Öörni et al., 1997). Oxidation releases fatty acids from phospholipids and, as a result, creates lysophospholipids (Steinbrecher 1984). Oxidation of cholesterol or CEs creates oxysterols such as 7-ketocholesterol, 5,6-epoxycholesterol, and 7-hydroxycholesterol, the most abundant oxysterols in human LDL being 27-hydroxycholesterol and 7-ketocholesterol (Hulten et al., 1996; Zhang, Basra, and Steinbrecher, 1990). 7-ketocholesterol activates CE formation, and thus foam cell formation while 7-hydroxycholesterol cholesterol inhibits cholesterol esterification and foam cell formation (Zhang, Basra, and Steinbrecher, 1990). Oxysterols have been found to contribute to inflammatory responses in tissues. Oxysterol 24-hydroxycholesterol, which mouse bone marrow macrophages secrete in response to Toll-like receptor activation (Diczfalusy et al., 2009), can affect inflammatory signaling in mice by inducing secretion of IL-6 (Gold et al., 2014). Also 4-HNE and 27-hydroxycholesterol are shown to induce expression and secretion of inflammatory cytokines TNF-a, IL-1β, and IL-8 in experiments with human promonocytic cell line U937, and the cells also produced matrix metalloproteinases upon activation by oxysterols (Gargiulo et al., 2015). Oxidized sterols can also reduce the activity of 3-hydroxy-3-methylglutaryl coenzymeA reductase (HMG-CoA reductase), the rate-limiting enzyme in cholesterol synthesis, thus reducing cellular cholesterol synthesis (Brown 1975), and they are also able to decrease the expression of lipoprotein lipase in human monocyte-derived macrophages as LPL mRNA is downregulated by oxysterols (Hulten et al., 1996).

In vitro, LDL can be oxidized by a selection of oxidative agents available. Transition metal ions, like Cu^{2+} (Ray 1954) are potent oxidative agents *in vitro*, but they lack clinical significance. Other oxidative agents that can oxidize LDL *in vitro*, such as peroxides secreted by endothelial cells (Steinbrecher et al., 1984), cigarette smoke ingredients (Yokode et al., 1988), myeloperoxidase in combination with H_2O_2 (Podrez et al., 1999; Savenkova, Mueller, and Heinecke, 1994), and the heme liberated from red blood cells (Miller, Altamentova, and Shaklai, 1997) are also relevant in atherosclerosis *in vivo* (Stocker and Keaney, 2004).

Plasma LDL transports several lipid-soluble antioxidant molecules that prevent oxidation of LDL in the circulation, such as α- and γ-tocopherol (vitamin E), lycopene, β-carotene, of which α-tocopherol is the most abundant (Esterbauer et al., 1991). These are consumed in the presence of oxidative agents, such as cigarette smoke, and eventually the lipids in LDL become oxidized (Valkonen and Kuusi, 1998).

Proteolysis of lipoproteins

The lipoproteins may become proteolyzed after they have entered the arterial wall or the aortic valve. Several different proteases are able to hydrolyze the protein moieties of lipoproteins. The neutral proteases plasmin, thrombin, and kallikrein are mainly derived from plasma (Piha, Lindstedt, and Kovanen, 1995), while cathepsin G is secreted by tissue resident macrophages (Wang et al., 2014), by neutrophils (Owen and Campbell, 1998) and by mast cells (Mäyränpää et al., 2006). Neutral mast cell proteases chymase and tryptase are also found in the arterial wall (Kovanen, Kaartinen, and Paavonen, 1995). In addition to the neutral proteases, acidic proteases, that are able to hydrolyze apoB-100 and other apolipoproteins, are found in the arterial wall. Cathepsins D (Hakala et al., 2003), F (Öörni et al., 2004), H (Han et al., 2003), K, and S (Sukhova et al., 1998) have been described to be present in atherosclerotic arterial wall. In stenotic aortic valves, mast cells (Wypasek et al., 2013), and hence the mast cell neutral proteases chymase, tryptase and cathepsins G are expressed in the diseased valves (Helske et al., 2006a). Moreover, the elastolytic cathepsins S, K, and V have been found also to be present in stenotic aortic valves (Helske et al., 2006b).

Hydrolysis of apoB-100 of LDL particles by different proteases can induce their fusion. Piha et al (Piha, Lindstedt, and Kovanen, 1995) found that if the generated apoB-100 fragments are not released from the proteolyzed LDL particles, but remain particle-bound, as occurs after treatment of LDL with the plasmin, kallikrein, or thrombin, the particles do not fuse, while hydrolysis of apoB-100 by α-chymotrypsin (Piha, Lindstedt, and Kovanen, 1995) or mast cell proteases (Kovanen and Kokkonen, 1991) resulted in release of the formed peptides, and fusion of the proteolyzed LDL-particles. By hydrolyzing LDL and VLDL with cathepsin D, Chen et al found that apoB-100 in these lipoproteins have different amino acid sequences available for proteolysis (Chen et al., 1991). Hydrolysis *in vitro* with α-chymotrypsin slightly increases the size of the particles and their affinity of LDL-particles to proteoglycans (Öörni et al., 2005). The specificity of α-chymotrypsin is very similar to that of chymase, an important mast cell protease which is capable of hydrolyzing apoB-100 (Kovanen and Kokkonen, 1991). Cathepsins D (Chen et al., 1991), F, B, and to a lesser extent, S, and K have been to observed to increase the size of the LDL particles, as well as their aggregation, fusion, and their affinity to proteoglycans (Öörni et al., 2004). In the arterial intima, macrophages and smooth muscle cells secrete these proteases.

Combinations of modifications

Single modifications of LDL have been described to enhance LDL uptake by macrophages in vitro (Pentikäinen et al., 2000). In vivo, usually several lipolytic or proteolytic enzymes, as well as oxidating agents, may be available, and the lipoproteins present may get multiply modified. Schissel (Schissel et al., 1996) isolated lipid particles from aortic intima and showed with *in vitro* -experiments that the particles were susceptible to hydrolysis by SMase, and that LDL hydrolyzes and aggregates more potently if LDL-particles were first oxidized or hydrolyzed with another lipolytic enzyme, PLA_2 (Schissel 1998). Chao (Chao et al., 1992) observed that trypsin pretreatment of native LDL, but not of oxidized LDL, was required for the CEs in the particle core to be hydrolyzed with cholesterol esterase. The reason for the oxidative modification to allow cholesterol esterase to act without prior proteolysis likely derives from the fact that also oxidation degrades apoB-100 and actually renders the surface of LDL particles unstable. E-LDL, an enzymatically multiply modified form of LDL, was immunohistochemically shown to be present in the arterial wall (Torzewski et al., 2004). E-LDL has been generated in vitro by treating LDL with trypsin and CEase to

mimic the lipid particles found in the intima. Trypsin was used in the model instead of matrix metalloproteinases (MMP) 2 and 9, that can degrade apoB-100 and make the LDL-particles more susceptible to macrophage uptake (Torzewski et al., 2004). E-LDL was found to induce foam cell formation in a mouse model (Chellan et al., 2016). Similarly, proteolysis combined with CE hydrolysis with LAL leads to enhanced aggregation of LDL and hence also enhanced uptake by macrophages (Hakala 2003). Plihtari and co-workers hydrolyzed LDL with several different proteases, chymase, plasmin, and cathepsin S that are found in the intima, and α-chymotrypsin, in combination with SMase and PLA_2-enzymes, and showed that proteolysis enhanced phospholipolysis of the particles, but induced fusion of the particles only when the peptide fragments were released from the lipoprotein particles (Plihtari et al., 2010). Thus, proteolysis with cathepsin S and chymase enhances lipolysis by PLA_2-V and SMase, and proteolysis before lipolysis can enhance the lipoprotein retention to proteoglycans (Plihtari et al., 2010). While not modifying LDL in the strict meaning of the word, amino acid exposure in apoB-100 is changed as LDL binds to the glycosaminoglycans. The binding to the glycosaminoglycans increases the exposure of segments that contain arginines and lysines on the surface of LDL, and also changes the organization of the core and the surface of the lipoprotein particles (Hakala et al., 1999). After LDL has been in contact with glycosaminoglycans, such as heparin, it is more potently hydrolyzed with PLA_2, and is susceptible to fusion and aggregation(Hakala et al., 1999). Finally, subsequent *in vitro* -experiments of treating LDL with cholesteryl esterase following with SMase (Guarino, Tulenko, and Wrenn, 2004) have shown that cholesterol crystals can be generated when LDL-particles are multiply modified.

Fate of modified lipoproteins

Extracellular lipid particles

In 1967 Smith and her co-workers (Smith, Evans, and Downham, 1967) showed that fatty streaks contained more lipid than normal intima and even more than advanced and fibrotic plaques. Lipid content of the intima reflects the source of the lipids: the CEs from "intima containing mainly perifibrous lipid" are rich in linoleic acid and the CEs from intima that contain foam cells are rich in oleic acid (Smith, Evans, and Downham, 1967). Lesions, called as "fibrous plaques" by Smith et al, rich in perifibrous lipid were found to contain unesterified cholesterol, and the early lesions with perifibrous lipid contain as much as 25 % of free cholesterol (Smith, Slater, and Chu, 1968). The proportion of unesterified cholesterol increases in the lesion as the proportion of linoleic acid decreases, and they suggest that the increase of free cholesterol is due to extracellular CE hydrolysis. Another type of lesion, described by Smith and co-workers (Smith, Slater, and Chu, 1968) as "larger plaques with amorphous centres", contain as much as 44 % of free cholesterol in their amorphous centers while the proportion of oleic acid decreases. Chung and co-workers (Chung et al., 1994) characterized also the protein composition of aortic lipoprotein-like particles from human arteries. They showed with SDS-PAGE that the lipid particle material from human aortic plaques contain apolipoproteins B, E, A-I, and C's, similarly to plasma VLDL, but that in the advanced human plaques immunoreactive apoB had disappeared. Chung and co-workers suggest in their paper that the lipid particles would be derived from TG-rich lipoproteins and would be surface remnants generated by lipolysis due to the apoA-I and apoC-content of the particles.

By homogenizing human aortas having normal intimas or atherosclerotic plaques, derived from autopsy samples, Hoff et al isolated lipoprotein particles that were similar to plasma LDL (Hoff et al., 1979), and showed that the isolated particles contained apoB-100. Following that, Hoff and Gaubatz (Hoff and Gaubatz, 1982) determined the chemical composition of aortic extracellular lipid particles and found that isolated lipids have similar or lower density than plasma LDL. Unesterified cholesterol content is increased in the particles isolated from a plaque, when compared to normal intima and plasma (Hoff and Gaubatz, 1982; Hoff, Pepin, and Morton, 1991). Hoff's work also shows that the fatty acid composition of the isolated lipid

particles is different in different stages of atherosclerosis (Hoff and Gaubatz, 1982). Guyton and Klemp (Guyton and Klemp, 1994)analyzed several human aortae fatty straks and plaque core and cap regions for free cholesterol and CEs. Their analysis showed that the oleate-enriched composition of the isolated lipid particles can be a marker of heavy deposit of lipid laden foam cells, in which the lipid particles contain mostly cholesteryl oleate, and that when the isolated lipids are linoleate-enriched, it can be a marker of mostly LDL-derived extracellular lipid, since LDL particles are enriched with cholesteryl linoleate (Guyton and Klemp, 1994; Smith, 1974). They calculated specific ratio (cholesteryl oleate/cholesteryl oleate+cholesteryl linoleate,) that describes the proportion of oleate-rich or linoleate-rich CE content and thus the origin of the extracted CEs. For comparison they calculated the same ratio from lipids that were extracted from LDL (0.32). In the fatty streaks, areas heavy with foam cells, the CE ratio was 0.8, showing heavy deposit of intracellular lipid on the area. In thevlipid deposits of the fibrous plaque and fibrolipid core the ratio was, in average 0.27—0.47, and from these results Guyton and Klemp suggest that both in the early and mature core regions, the main proportion of the lipid is derived from plasma lipoproteins as the CEs had a low oleate/linoleate ratio (Guyton and Klemp, 1994). In contrast to the core regions, however, their results showed that cap regions of these advanced plaques had high oleate/linoleate ratio, suggesting that these areas were rich in foam cells and intracellular lipid droplets derived from them (Guyton and Klemp, 1994).

Kruth and co-workers (Kruth, 1984) described the distribution of extracellular CEs and unesterified cholesterol with Oil Red O and filipin-stainigns performed on human aortic tissue. Unesterified cholesterol is found in the extracellular compartment and esterified cholesterol, stained with Oil Red O, is found in the cells, and occasionally also in the extracellular compartment. Also cholesterol crystals were seen in the extracellular space. Shortly after Kruth and co-workers, Simionescu and her co-workers (Simionescu et al., 1986) showed with electron micrographs that radiolabeled β-VLDL accumulated in aortic extracellular matrix of a cholesterol-fed rabbits (Simionescu et al., 1986). They named these structures "extracellular liposomes" and demonstrated liposome-like and multilamellar structures, rich in unesterified cholesterol. Similar structures are seen in rabbit aortic valves after a high fat diet (Simionescu et al., 1986).

Pasquinelli and co-workers (Pasquinelli et al., 1989), examined several human carotid artery intimas and demonstrated multilamellar vesicles among smaller lipid particles in all stages of atherosclerosis. In their study, extracellular lipid particles found in the intima were larger than native LDL but smaller than intracellular lipid particles. Uni- and multilamellar lipid-containing vesicle-like particles were found also in the human and rabbit aortas by Chao and co-workers (1988) and by Guyton and Klemp (1989). Also the particles later isolated by Chao from where (Chao et al., 1990) showed multilamellar vesicle appearance. Chao and co-workers isolated extracellular lipids from human abdominal aorta samples and by aid of centrifugation divided them into unesterified cholesterol-rich and esterified cholesterol-rich categories (Chao et al., 1990). Results of Chao and co-workers showed that the particles are smaller (< 100 nm) than the intracellular lipid droplets (300 – 6000 nm). Also their lipid analysis results showed that the lipid particles from extracellular compartment of an aorta have different lipid characteristics compared with LDL-particles and their lipid content, as shown in Table II below.

Table II Lipid characteristics of extracellular lipid particles of human abdominal aorta
TC = Total cholesterol, PL = phospholipid, UC = unesterified cholesterol, SM = sphingomyelin, PC = phosphatidylcholine.

	TC/PL	UC/TC	UC/PL	SM/PC
LDL	3.1	0.38	0.67	0.4
CE-rich	10.1	0.24	2.5	1.7
UC-rich	3.2	0.77	2.6	1.9

(Chao et al., 1990; Esterbauer et al., 1992)

Accumulation of lipids in the arterial intima starts at very early age (Ylä-Herttuala et al., 1986). Ylä-Herttuala and co-workers studied arteries of Finnish children, and showed that the concentration of arterial CEs increases from birth to 15th year at a constant rate, and that the constant-level content of cholesteryl oleate suggests that the lipid is derived directly from lipoproteins and not synthetized in the arterial wall. Later, Torzewski and co-workers studied aortas of a similar group of children by immunohistochemistry and histological stainings for proteoglycans and lipid-rich areas. According to their findings, in arteries of children below 1 year, the intima was thin and contained ample amounts of apoB-100, however they found only a few lesions that stained with lipophilic Sudan IV-stain. In the aortas of children between 1–5 years, intima was already thickened, but very little apoB-100 was found, and no lipid rich areas staining with Sudan IV-stain were found. In aortas of the children from age 5 to 15, apoB-100 was found also in much deeper layers of thickened intima (Torzewski et al., 2009). In an older group of patients (ages 7—49), Nakashima and co-workers (Nakashima et al., 2007) showed with immunohistochemistry for apoB-100 and lipid stainings that the extracellular lipid starts to accumulate deep in the intima near the internal elastic lamina already in very early stages of atherosclerosis. Only later, when the fatty streaks with heavy intracellular lipid accumulation start to develop, macrophages enter the scene (Nakashima 2007). The heaviest lipid accumulation is found in the atheroma, and at this stage, also macrophages are found close to the deep lipid accumulation site (Nakashima 2007).

Cholesterol crystals

Unesterified cholesterol is derived from enzymatic hydrolysis of esterified cholesterol from the core of lipoprotein particles. It can also be derived from cholesterol-rich erythrocyte plasma membranes, after extravasation of erythrocytes from leaky neovascular capillaries in an advanced atherosclerotic plaque (Virmani et al., 2005), and from cholesterol-containg lipid droplet-loaded macrophages, which are able to transfer unesterified cholesterol to extracellular space (Kruth et al., 2001; Ong et al., 2010). Unesterified cholesterol will form crystals when it reaches the critical concentration (Katz, Shipley, and Small, 1976; Konikoff et al., 1992). The critical concentration of cholesterol depends on other lipids available: if the solution contains enough phospholipids, they help solubilizing cholesterol up to 33 mol % concentration in aqueous solutions, and CEs can solubilize cholesterol up to 8 mol % (Katz, Shipley, and Small, 1976). Guarino and co-workers demonstrated that cholesterol crystals can be generated in vitro from lipoprotein particles by pretreating LDL first with cholesteryl esterase and then hydrolyzing it with SMase (Guarino, Tulenko, and Wrenn, 2004). They also suggest that a prerequisite for crystallization is LDL aggregation, as a nucleation center is required for crystals to form. In atherosclerotic lesions, cholesterol crystals can exist both intracellularly (Klinkner et al., 1995; Pasquinelli et al., 1989; Tangirala et al., 1994) and extracellularly (Bocan, Schifani, and Guyton, 1986; Guyton and Klemp, 1989). In the intracellular compartment, that is, in the foam cells, the cholesterol containing lipoproteins and modified lipoprotein-derived particles that the cells have taken up, are hydrolyzed in the lysosomes by LAL (Brown, Ho, and Goldstein, 1980).

Fatty streaks seldom contain cholesterol crystals, while in the more severe lesions they are frequently found (Katz, Shipley, and Small, 1976). While in atherosclerotic arterial wall the cholesterol crystals are plentiful (Figure 13), in human stenotic aortic valves they are perhaps not found as often as in atherosclerotic arteries (Foreman and Fulkerson, 1987; Kuusisto et al., 2005). However, in bioprosthetic valves crystals may develop in the course of time: Price and co-workers presented a case, where a bioprosthetitc aortic valve was replaced after 21 years, and the leaflets were filled with crystallized cholesterol (Price et al., 2007). In animal models of atherosclerosis, however, aortic valves are shown to develop cholesterol crystals (Duewell et al., 2010).

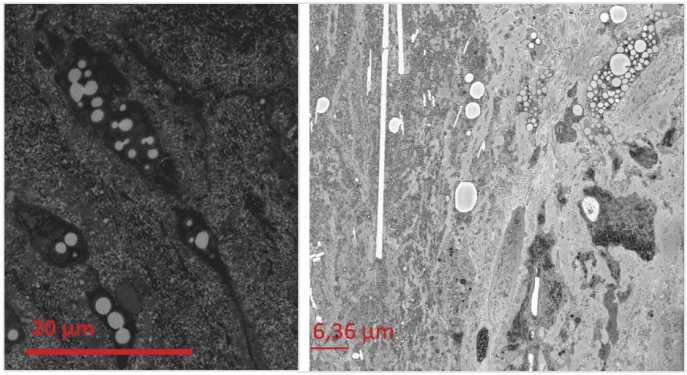

Figure 13. Foam cells and cholesterol crystals in a human carotid artery intima. Foam cells are lipid droplet filled cells that can be derived from macrophages or smooth muscle cells.

Foam cells

Extrahepatic cells take up native LDL via LDL receptors, which are downregulated in the presence of excessive LDL (Brown, Dana, and Goldstein, 1973; Goldstein and Brown, 1977) (Figure 14). Thus, this physiological uptake does not lead to foam cell development. Macrophages have another set of receptors for clearing oxidized, modified, and damaged lipoproteins (Figure 14). Scanvenger receptors, CD36 SR-A and SR-B recognize oxidized and enzymatically modified LDL and sequester it into lysosomes for degradation. However, as shown in the thin inflamed walls of human intracranial arterial aneurysms, partly undegraded oxidized LDL is retained in the lysosomal compartment or other intracellular compartments of the macrophages suggesting that oxidized LDL does not degrade well in the lysosomes (Ollikainen et al., 2016), as cholesterol in oxidized LDL is not accessible to re-esterification by the cytoplasmic acyl-CoA:cholesterol acyltransferase-enzyme (ACAT) (Zhang, Basra, and Steinbrecher, 1990). While uptake of native LDL by LDL receptor is downregulated by high intracellular cholesterol concentration, uptake via scavenger receptors is not inhibited by the internalized cholesterol. The receptors also scanvenge otherwise modified lipoproteins, which become quickly degraded in the lysosomes. Resulting unesterified cholesterol is either transported for use of membranes or released from the cell. The unesterified cholesterol that is not used for the cell can be effluxed to HDL for example via ATP-binding cassette transporters A1 and G1 (ABCA1 and ABCG1) (Lee-Rueckert, Escola-Gil, and Kovanen, 2016). Since accumulating unesterified cholesterol would induce apoptosis of macrophages (Yao and Tabas, 2000), unesterified cholester in the cell is re-esterified by acetyl-coenzyme A acetyltransferase (Brown, Ho, and Goldstein, 1980) and it is stored as CE-containing intracellular lipid droplets (Figures 13 and 14). ACAT has a preference for oleic acid as the fatty acid substrate (Chang et al., 2010), and so the intracellular lipid droplets contain largely cholesteryl oleate. Also the protein moiety of lipoproteins, apoB-100, is degraded by lysosomal proteases such as various cathepsins (Linke et al., 2006) and the liberated individual amino acids and small peptide fragments are transported to the cytosol. The degradation of apoB-100 is inhibited in mouse macrophages *in vitro* by oxidized LDL phospholipids, which results in filling of the lysosomes with only partially degraded apoB (Itabe et al., 2000). *In vitro* experiments have shown that variously modified lipoproteins are extremely susceptible to be taken up by macrophages (Aviram and Maor, 1992; Hoff, O'Neil, and Cole, 1991), as are the remnant lipoproteins (Elsegood et al., 2001; Yu and Mamo, 2000). Foam cells can be generated both from macrophages and smooth muscle cells (Allahverdian, Pannu, and Francis, 2012). In smooth muscle cells cholesterol efflux is less effective than in macrophages as ABCA1 is less expressed in smooth muscle cells compared to myeloid-origin cells (Allahverdian, Pannu, and Francis, 2012). Also dendritic cells have been shown to accumulate lipid droplets in hypercholesterolemic mice (Paulson et al., 2010).

Stary describes foam cell formation as a key feature of a "fatty streak" in atherosclerotic arteries (Stary et al., 1994a). Foam cells start accumulating in the intima only after the lipoprotein-derived lipids already have accumulated in the intima (Nakashima et al., 2007). The density of macrophages increases as the intima thickens. Foam cells are found also in the stenotic aortic valves, although at a lesser density distribution than in the atherosclerotic artery intima (Otto et al., 1994; Syväranta et al., 2014). Both macrophage-derived and smooth-muscle cell derived foam cells are found in atherosclerotic lesion (Allahverdian, Pannu, and Francis, 2012) and in the stenotic valves (Latif et al., 2015). Lysosomal degradation of ox-LDL in cultured mouse macrophages and in a human cell line THP-1-macrophages is compromised compared to that of acetyl LDL or native LDL (Hoff et al., 1993; Yancey and Jerome, 2001). Also the lectin-like oxidized LDL receptor LOX-1 is expressed in macrophages and mediates the uptake of oxLDL by these cells (Pirillo, Norata, and Catapano, 2013).

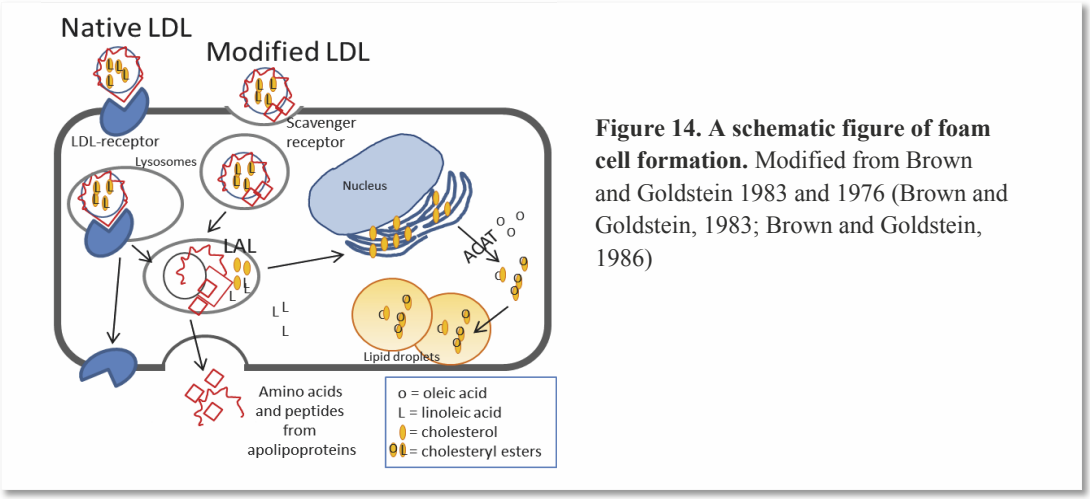

Figure 14. A schematic figure of foam cell formation. Modified from Brown and Goldstein 1983 and 1976 (Brown and Goldstein, 1983; Brown and Goldstein, 1986)

Inflammation in atherosclerosis and aortic stenosis

Inflammation is considered as a degenerative factor atherosclerosis. Initially, macrophages perform reparative measures: they start clearing excessive extracellular lipid. However, because this is an overwhelming task, a vicious circle ensues and inflammation starts to deteriorate the intima. As Nakashima showed,macrophages only enter the intima after lipid has already accumulated and retained in the aortic wall (Nakashima et al., 2007) and inflammation is likely a response to retention of lipoproteins and their modifications. Inflammatory cells, such as T-cells (Zhou, Stemme, and Hansson, 1996), macrophages (Nakashima et al., 2007; Olsson et al., 1994), and mast cells (Kaartinen, Penttila, and Kovanen, 1994) accumulate in the thickened intima that is filled with extracellular lipid particles. The modified, oxidized or lipolyzed lipoproteins can attract T-cells (McMurray, Parthasarathy, and Steinberg, 1993) and monocytes (Quinn, Parthasarathy, and Steinberg, 1988). Macrophages and T-cells secrete cytokines, such as IL-1β, IL-18, and γ-interferon, which can induce remarkable rearrangement of the intimal tissue by activating resident smooth muscle cells or endothelial cells to produce extracellular matrix (Pober and Tellides, 2012), or by inducing secretion of enzymes that can degrade the collagen and elastic laminae in the inflamed atherosclerotic lesions (Galis et al., 1995; Galis et al., 1994). Matrix remodeling in aortic valve leaflets it is driven by mast cell-derived proteases (Helske et al., 2006a), MMPs (Kaden et al., 2005a), and cytokines like TNFα that increase cell proliferation (Kaden et al., 2005b). Macrophages and T-cells start to accumulate already in early stages of aortic stenosis (Otto et al., 1994).

In aortic valves IL-1β has been shown to activate aortic valve interstitial cells into an inflammatory phenotype (Kaden et al., 2005a; Kaden et al., 2003; Nadlonek et al., 2013). Bone formation in stenotic aortic valves is associated with inflammatory cell accumulation, and T- and B-cells and mast cells are found in the same areas with lamellar bone, so providing a link between inflammation and calcification at the cellular level (Helske et al., 2004; Mohler et al., 2001; Olsson et al., 1994). IL-1β has been found to stimulate MMP-expression in macrophages: Stimulation of collagenolytic MMP1 production by endothelial cells may lead to decreased content of collagen in the shoulder of an atherosclerotic plaque (Huang, Mironova, and Lopes-Virella, 1999) and thus weaken the plaque stability and functionality.

Several lipid hydrolysis products are proinflammatory. LPC, a product of phospholipid hydrolysis, can induce IL- 1β secretion by monocytes (Liu-Wu, Hurt-Camejo, and Wiklund, 1998) and upregulate the expression of adhesion molecules on the endothelium (Kume and Gimbrone, 1994), and so contribute to the accumulation of inflammatory cells. Free fatty acids are found to amplify inflammation in monocytes, especially in type 2 diabetes, and this they do by increasing expression of toll-like receptors (TLRs), nuclear factor- κB, monocyte chemoattractant protein-1 and IL-1β (Dasu and Jialal, 2011). Cholesterol crystals have recently been found to possess proinflammatory potential, as they can activate the inflammatory complex called inflammasome in macrophages and thereby induce IL-1β-secretion by these cells (Duewell et al., 2010; Rajamäki et al., 2010)

Inflammasome

Innate immunity is a first-in-line defence system that immune system cells use against any harmful stimuli. Such stimuli can be pathogens, dead cells and cell debris, and various irritative agents or foreign bodies. Inflammasome is considered as a driving factor in many autoimmune conditions, such as in gout and rheumatoid arthritis. In the innate immunity cells, there is a censor system, that consist of cell membrane receptors and an intracellular protein complex called inflammasome. The inflammasome consists of multiple proteins that form a large complex when activated. Several types of inflammasomes have been described, but NLRP3-inflammasome complex is the most relevant one for atherosclerosis. The core of the complex is NLRP3-protein (nucleotide-binding domain leucine-rich repeat containing (NLR) family, pyrin domain containing 3) which complexes with adapter protein (apoptosis-associated speck-like protein containing caspase activation and recruitment domain (ASC) (Stutz et al., 2013). NLRP3-complex recruits procaspase-1 which then becomes cleaved to mature caspase-1, which, in turn, is able to cleave pro-IL-1β and pro-IL-18 into their biologically active forms that can be secreted (Ghayur et al., 1997; Thornberry et al., 1992). Inflammasome requires two signals for its activation. The first one, a priming signal, (Latz, Xiao, and Stutz, 2013) is mediated via pattern recognition receptors, such as the Toll like receptors (TLRs), that recognize either pathogen associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs), (Bauernfeind 2009). The primining signal induces transcription of NLRP3 and of pro-IL-1β or pro-IL-18. For inflammasome complex build-up and subsequent cytokine cleavage, a second stimulus is required. The second stimulus can be an endogenous one, like extracellular ATP derived from damaged cells (Ferrari 1997), acidic pH (Rajamäki et al., 2013), or cholesterol crystals in the arterial intima (Duewell et al., 2010; Rajamäki et al., 2010), or it can come from outside of the body, like asbestos (Sayan and Mossman, 2016) or extremely fine carbon particles (Murphy et al., 2012). Oxidized LDL has been suggested to activate inflammasome as Duewell et al demonstrated that it induced IL-1β -secretion by inflammasome activation in a mouse model (Duewell et al., 2010). NLRP3 inflammasomes have been found in human atherosclerotic coronary arteries (Rajamäki et al., 2016) and carotid arteries (Shi et al., 2015), and the levels of expression of the key NLRP3 inflammasome components correlate with the severity of atherosclerosis.

Aims of the study

The extracellular lipid in aortic stenosis and atherosclerosis has been considered as a rather unreactive debris that accumulates and is cleared by macrophages. Also, the spatial distribution of diffeent lipid groups in the extracellular lipid has been unknown. This gave the rationale to examine the extracellular lipid more closely.

More specifically, the aim was to investigate the following:

1. To examine the spatial distribution of different classes lipids and species therein in the intima, and to visualize the spatial arrangement of cholesterol crystals and the lipid species in atherosclerotic arterial intima and the underlying media.

2. To isolate and characterize the extracellular lipid particles from stenotic aortic valves and from carotid atherosclerotic lesions in order to examine the chemical and physical properties as well as modifications of lipoprotein particles that may lead to generation of such particles.

3. To examine the effect of the extracellular lipid particles on inflammasome activation in macrophages.

Methods

Table III. Methods used in the studies

Method used	Study	
Mass spectrometry, ESI-MS	**I, III**	
Mass spectrometry, ToF-SIMS	**II**	
Liquid chromatography-mass spectrometry	**III**	
Thin layer chromatography	**I**	(Lähdesmäki et al., 2009)
Electron microscopy, 3-view	**III**	(Belevich et al., 2016)
Electron microscopy, tomography	**III**	(Mastronarde, 2005)
Electron microscopy, size analysis	**I**	
Circular dichroism	**III**	
Dynamic light scattering	**I, III**	
Rate zonal ultracentrifugation	**I, III**	
Western blot	**I, III**	
Lipid extraction	**I, II, III**	(Folch, Lees, and Sloane Stanley, 1957)
Cell culture, macrophages differentiated from human primary peripheral blood monocytes	**II, III**	(Nakanishi et al., 2009)
Statistical methods, multivariate analysis	**I,II,III**	
Immunohistochemistry		
ApoB-100	**II, III**	
ApoA-I	**III**	
ApoC-III	**III**	
ApoE	**III**	
CD31	**II**	
CD34	**II**	
CD163	**II**	
Glycophorin A	**II**	
ELISA		
ApoB-100	**I,III**	
Apo-E	**I**	
IL-1β	**III**	

Extracellular lipid particle isolation

Extracellular lipid particles were isolated from human aortic valves and human carotid arteries. The aortic valves were acquired from aortic valve replacement surgery and the carotid artery sections were from endarterectomy surgery. All patients had signed an informed consent form prior to the operation. For each isolation, one whole valve leaflet was used, and in case of carotid arteries, pieces of several carotid arteries were combined for a pool, which will be referred to as "sample" in the text. Prior to the isolation, tissues were stored at-80 °C. The tissues were ground in liquid nitrogen into fine particles with mortar and pestle, after which salt-based isolation buffer (0.01 M Tris, 0.15 M NaCl, pH 7.4 and 0.01 M Tris, 1.5 M NaC)

containing 0.02 mM butyrated hydroxytoluene, 0.1 % EDTA, 0.01 % NaN_3, and a protease mix was applied onto them. The sample was gently agitated in isolation buffer for 10 minutes, and centrifuged for 30 min at 10 000 g, +4 °C. The supernatant containing extracellular lipid particles was collected into a fresh tube (on ice) and high salt buffer (1.5 M NaCl) was applied onto tissue homogenate. After gently agitating the sample for 10 minutes, the tube was placed in an ultrasonic bath in ice water for 10 minutes to detach remaining lipid particles from the extracellular matrix. The samples were recentrifuged for 30 minutes at 10 000 g, +4 °C. The supernatants were collected and combined with the first supernatant.

The density of the sample was set to 1.063 with D_2O-based buffers (0.1 M Tris, 0.15 M NaCl in D_2O, *d* = 1.116 g/ml and 0.1 M Tris, 0.15 M NaCl in H2O, *d* = 1.006 g/ml, both containing 0.1 % EDTA) and ultracentrifuged for 24 h in a SW41Ti rotor (*gmax* 288 000 x g, 40 000 rpm) at +4 °C, and the lipid particles were collected from the top of the tube. The samples were stored at +4 °C until further analysis.

Plasma lipoproteins

Human plasma lipoproteins were isolated from plasma that was obtained from healthy volunteers (Finnish Red Cross Blood Service, Helsinki, Finland). Plasma VLDL (d < 1.006 g/ml), IDL (d = 1.006-1.019 g/ml), and LDL (d= 1.019-1.050 g/ml) vere isolated by sequential ultracentrifugation in the presence of 3 mmol/l Na_2EDTA (Havel, Eder, and Bragdon, 1955; Radding and Steinberg, 1960). EDTA and 100 µg/ml of gentamicin sulfate (Lonza, Basel, Switzerland) were added to plasma, and the plasma was centrifuged at 40 000 rmp (rotor 50.2 Ti, gmax 302 000) at +4 °C for 24 h. The VLDL fraction was collected from the top of the tube, and the density of the remaining plasma was set to 1.019 g/ml with KBr, and the density-adjusted plasma was then centrifuged at 40 000 rpm for 24 h. After that, IDL fraction was collected from the top of the tube. Density of the remaining plasma was set to 1.050 g/ml with KBr, and the density-adjusted plasma was centrifuged at 40 000 rmp for 24 h, after which the LDL fraction was collected similarly as the VLDL and IDL fractions. LDL fraction was "washed" by recentrifugintg it at a density of 1.063 g/ml. The collected top fraction of the tube was dialyzed against 1 mM EDTA-150 mM NaCl, pH 7.4, after which the protein concentrations of lipoproteins were measured by the method of Lowry (Lowry et al., 1951), using bovine serum albumin as standard.

Methods used in the thesis

Lipid analysis

Thin layer chromatography

Thin layer chromatography (TLC) separates lipid classes by their ability to bind to the stationary phase (silica plate) and to travel with the mobile phase, depending on their polarity. The "neutral" lipids such as free fatty acids, cholesterol, CEs and TAGs, that require adequate proportion of hydrophobic solvents for their mobility in the stationary phase, and the phospholipids, such as PCs, LPC s, and SMs require larger proportions of hydrophilic solvents in the mobile phase. If the degree of hydrophobicity of individual lipid species in one lipid class is large enough, the technique is able to separate the species, provided that suitable mobile phase and long enough travel time PC on the stationary phase are used. Usually it is enough to separate only the main lipid classes. Modern High Performance TLC (HPTLC) allows quantitative analysis if a suitable set of lipid standards is used. Thus HPTLC serves well in quantification of the proportions of different lipid classes, and particularly the proportions of unesterified and esterified cholesterol. Alcoholic cholesterol does not ionize well and would require derivatization prior to lipid mass spectrometry, and therefore, in this thesis, HPTLC was an useful technique in measuring the proportion of unesterified cholesterol in the mixture of tissue lipids. From the same samples, it was possible to gain information of the ratio of glycerophospholipids to cholesterol. However, to gain more detailed information of different species within lipid classes, another analysis method was needed.

Mass spectrometry

While HPTLC was an useful method in analyzing the proportions of different groups, mass spectrometry provided detailed information of different species within lipid groups. In this thesis, two different mass spectrometry methods were used: electrospray ionization (ESI) to analyze lipids and proteins and secondary ion mass spectrometry to analyze lipids on the cryosection surfaces.

In mass spectrometry, analytes are given either a positive or negative charge by ionization and separated by their mass and charge (m/z) ratio. The most electronegative molecules or side chain groups are the most easily ionized. Unorganic molecules are ionized according their capability to accept or lose protons/electrons. Lipids usually acquire or lose only one electron while proteins can get several charges, depending on their composition.

Electrospray ionization

In electrospray ionization (ESI) the analytes in organic solution are sprayed through a narrow capillary in a high voltage field gas and the formation of the fine spray is supported by hot gas stream. The sprayed analytes in the liquid droplets become charged, and as the solvent evaporates, charges densify, and repel each other, and optimally only the charged analytes end up in the vacuum chamber and to the mass analyzer. (Figure 15). ESI-mass spectrometry (ESI-MS) is considered as a soft ionization method and thus is suitable for lipids since it does not extensively fragment the lipids (Brügger et al., 1997). By using suitable standards ESI-MS can be used for a semiquantitive analysis. True quantification requires numerous lipid standards with acyl chains having different chain lengths and degree of unsaturation stages. The ions can be either protonized or deprotonized to form positive or negative ions, respectively (Thomson and Iribarne, 1979). ESI can be combined with different mass analyzers, for example ion trap, quadrupole, and time of flight (TOF)-analyzer

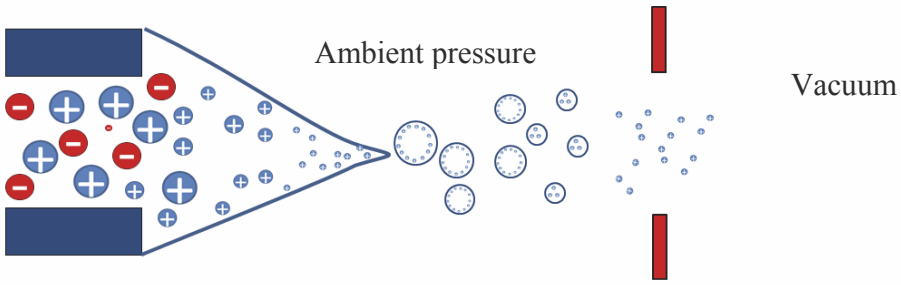

Figure 15. Electrospray ionization. The tip of the ionization probecapillary which is at positive voltage, and from which the positively ionized analytes are repelled with the solvent. Droplets of solvent containing the ionsare reduced in size due to evaporating gas stream and in optimal case the ionized analytes are emitted and carried into the mass analyzer parts in vacuum.

The structural characteristics of lipids affect their ionization: PC, SM, CE, and TAG all can be charged positively when ammonium is supplied, and thus are analyzed in the positive ion mode. Fatty acids, PS, and PI rather lose protons and are analyzed in the negative ion mode. The PE is zwitterionic and both ionization modes can be used. In ESI-MS, the efficiency of ionization drastically depends on the lipid chain lengths and degree of unsaturation (Brügger et al., 1997). For example, even if equimolar amounts of two PCspecies are administered to the mass spectrometer, species with the shortest acyl chains or with the highest degree of unsaturation gain the highest spectral peak intensities (Brügger et al., 1997). The same applies to CE, SM, and TAG species. Hydrophobic, or nearly hydrophobic lipids, such as unesterified cholesterol with a large bulk of nonpolar sterol backbone, ionize poorly and are not readily detectable with ESI-ionization mass spectrometry without derivatization (Liebisch et al., 2006). To enhance ionization of lipids, their different derivatives or adducts can be used; in ESI-MS ammonia is frequently added to produce ammonium-adducts (Duffin 1991, Crawford 1986).

TOF-SIMS

Time of Flight Secondary Ion Mass Spectrometry (TOF-SIMS) was originally used to analyze surfaces in metallurgy. Recently, it has also been adapted to analyze biological samples, especially lipids, as the mass-charge ratio range of TOF-SIMS is well suited to small molecules and their fragments. In this thesis TOF-SIMS was used to analyze spatial distribution of lipids of cryosections of coronary arteries, as with SIMS methods, analysis directly from surface is possible. Originally only the secondary ions from smallest molecules like amino acids, drugs, vitamins, and small peptides could be collected (Benninghoven and Sichtermann, 1978), but later when the energy of the primary ions could be adjusted to better suite organic samples, also larger molecules, such as lipids could be detected (Walker, 2008). In the SIMS, samples are placed in a vacuum and bombarded with multiatom primary particles consisting of bismuth (Bi_n^+), gold (Au_n^+) or fullerene (C_{60}^+) to desorb the outmost molecule layer of the tissue surface (Benninghoven, 1994; Passarelli and Winograd, 2011; Walker, 2008). The detached material - secondary ions - are then collected into the mass analyzer. A full mass spectrum can be acquired from each analyzed voxel, with sub-micrometer resolution. As each primary ion beam only erodes the surface by depth of 1-2 nm, the analysis can be repeated, so that both positive and negative ion spectra can be collected from every voxel. When the mass spectra are collected sequentially in a grid of voxels, by combining the spectra from every voxel, it is possible to create surprisingly detailed images of spatial distribution of any analyte on the surface, and both

the mass spectra and the image generated from the combinations of several different spectra can be used to obtain even more detailed information of the samples. Initially, the images were two-dimensional, but as the primary ion sources developed into less damaging cluster ions, also three-dimensional imaging has become possible, as several layers of a sample can be imaged (Walker, 2008).

TOF-SIMS is hardly a method easily accessible for every laboratory, as the equipment is expensive. The samples must be sectioned and mounted on special conductive silica plates and thoroughly dehydrated, but otherwise no extensive preparation is necessary. Target analytes break due to the high energy impact of the primary ion. Rhe sensitivity of SIMS is reduced at over m/z 1000, due to molecule fragmentation or poor ionization of large molecules. Therefore,TOF-SIMS suitsbest for small molecules, such as lipids, and is less suitable for studying proteins with high mass (Chughtai and Heeren, 2010; Passarelli and Winograd, 2011). In lipid ToF-SIMS the challenges are similar to those in ESI-MS-analysis. Lipid species that aquire or lose protons easily, as many phospholipids do, tend to ionize more easily than hydrophobic of nonpolar lipids, such as cholesterol CEs (Liebisch et al., 2006). Thus the intensities of phospholipids are seemingly higher than those of cholesterol and CEs, even if, for example in atherosclerotic arteries, the actual amounts of these neutral lipids is extremely high. Furthermore, in TOF-SIMS all ions are desorbed simultaneously, and so the matrix effects created by the better ionization efficiency of the phospholipids suppress the intensities of neutral lipids. This can be also seen when higher spatial resolution is selected for better image resolution: the smaller the voxel area is, the smaller is the amount of desorbed and ionized molecules, and the intensities of neutral lipids are suppressed even more than in the high mass resolution mode (Chughtai and Heeren, 2010; Passarelli and Winograd, 2011).

Protein analysis

Circular dichroism

To analyze the conformation of the protein moiety of the extracellular lipid particles, circular dichroism was used. Secondary structure of proteins consists of α-helices, β-sheets and random coils. In circular dichroism, these secondary structures are analyzed based on their ability to absorb and to turn polarized light in a plane (Beychok, 1966; Beychok, 1968). With circular dichroism it is possible to show the differences in the secondary structures: a protein consisting mainly of α-helix has a different spectrum from a protein rich in β-sheet or random coil (Beychok, 1966; Kelly and Price, 2000; Tiffany and Krimm, 1969). The method is useful in showing possible changes in protein structure under different conditions. Usually the structure of a protein is a combination of several different types of secondary structures, and, accordingly, circular dichroism spectrum is a combination of circular dichroic curves for the α-helix, β-sheet, and random coil conformations (Beychok, 1966; Greenfield and Fasman, 1969). In a mixture of proteins, such as the extracellular lipid particles measured in this thesis, all components contribute to the circular dichroism spectrum (Beychok, 1966), so for a mixture of different proteins and these other proteins may interfere or add to the spectrum (Beychok, 1968; Kelly, Jess, and Price, 2005). For analysis and to get reliable results of the protein structure, it is necessary to purify the protein from excess salt or chaotropic elements such as urea, since these compounds affect the structure of protein (Kelly, Jess, and Price, 2005).

Isolation and analysis of extracellular lipid particles

Isolation of the extracellular lipid particles

Isolating extracellular lipid particles from atherosclerotic tissues can be achieved by many different methods, depending on the required material quality. Common feature to the methods is that the tissue is minced into pulp in isotonic buffer containing EDTA. Several different methods have been used to homogenize the

tissues. In some studies tissues have been homogenized with an electronic homogenizer (Polytron) to obtain fine enough pulp to enhance sufficient lipid particle isolation (Hoff and Clevidence, 1987; Hoff and Gaubatz, 1982; Hoff and Morton, 1985). Others have relied on glass homogenizer (Li et al., 2005), tissue choppers (Ylä-Herttuala et al., 1988), or scissor mincing (Chao et al., 1988; Chao et al., 1990; Li et al., 2005). In this thesis tissue homogenization in liquid nitrogen was used. In this method the tissue was kept frozen under liquid nitrogen and it became thus brittle and was easily broken with mortar and pestle. Heavily calcified tissues, such as stenotic aortic valves, and sometimes also carotid arteries, can otherwise be difficult to homogenize. As the tissues are kept under nitrogen, also the effect of oxygen is minimized. The homogenization method has, however, less effect on the result than the next isolation step. That step determines the limits of the further analysis.

Isolation of the lipid particles from tissues can be carried out by affinity or size exclusion colums, or by a density gradient centrifugation. Affinity columns isolate particles containing a specific component, such as apoB-100, which, on the other hand, limits the gain to the particles in which the antigenicity has been preserved. Size exclusion or gel filtration columns limit the size of the particles acquired. The smallest particles flow through the column, and the pore size of the column defines the largest size that can be passed through. Ultracentrifugation of the tissue extracts separates the particles according to their density, which is dependent on the composition of the particles. (Redgrave, Roberts, and West, 1975). This method was used in this thesis, as for the analyses, all lipid particles that had lower density than 1.063 g/ml were collected so that no part of the extracellular lipid particles were excluded. The density gradient ultracentrifugation was also used to concentrate the extracellular lipid particle preparate. When the centrifugation procedure is carried out with KBr solutions, the samples may require dialysis against a low-salt solution. If dialysis is not suitable for the overall method, the density can be achieved also by using D_2O-based salt buffers in combination with H_2O-based salt buffers. In this thesis, D_2O-based buffer system was used in separation, as with D_2O, dialysis was not necessary. With minimizing the amount of purification steps, possible loss of sample was avoided.

Size analysis

Electron microscopy measurements

For measuring the size of the particles, several different methods are available. With electron microscopy images, sizes of the particles can be accurately measured (Hakala et al., 1999; Paananen and Kovanen, 1994); given the scale bar is correct (Forte 1972). From a thin section and a negative staining, only two dimensions of the particles are taken into account, i.e. the exact length of the cross section of the particles can be detected. If the particles in solution are applied to a grid and dried onto it, it is possible to try to detect aggregation of the particles. However, it is not possible to separate the genuine aggregates from artefactual aggregates generated during treatment of the samples.

Electron tomography and 3-view electron microscopy

Electron microscopic tomography (EM tomography) and 3-view EM are methods with which it is possible to obtain three-dimensional images of the ultrastructure of cell organelles and various tissue structures (Skoglund et al., 1986). For the EM tomography, the images are constructed from semi-thick sections (250 nm) that are cut out of resin-embedded blocks. The specimens are imaged and composed using a software to create three-dimensional images (Kremer, Mastronarde, and McIntosh, 1996; Mastronarde, 2005). The outlines of the particles are traced and the images are visualized using the MIB-software (Belevich et al 2016). In EM tomography, the semi-thick section is imaged from several different angles, which can bring out even the finest details of subcellular structures and even viruses. Here, tomography was utilized to verify

particle fusion. Detecting the actual fusion of the particles requires similar high resolution, as actual absence of any membrane between two apparently fusing particles has to be reliably detected. The 3-view EM is essentially scanning electron microscopy, where several hundred layers of a resin-embedded sample are scanned and combined as a stack with a software. With the 3-view EM, larger areas can be imaged than with tomography, but in process of sectioning the tissue in 40 nm intervals, some fine structures, such as the smallest lipoproteins, may be lost.

Dynamic light scattering

Measuring size of the particles within the size range of 10 to 1000 nm cannot be performed with visual methods except with electron microscopy. Unfortunately, sample preparation for electron microscopy is time consuming and demanding. Measuring of a large number of samples at one time is more efficient with dynamic light scattering (Sartor, 1970). This method relies on several photons scattering from a particle, and on detecting the diffusion rate of particles of different sizes (Goldberg, 1996). Small particles tend to move randomly more and faster than large particles do. The particles or particle aggregates rotate around, and the non-spherical pieces are approximated as spheres according to their largest dimension. This method does not separate large particles from aggregates. In the current study, dynamic light scattering was a suitable method to useas the sample can be recovered from the cuvette for future analyses, and multiple samples could be measured relatively fast.

Rate zonal ultracentrifugation

The size of the particles can be determined using a density gradient in rate zonal ultracentrifugation, where the particles float according to their respective sizes. Thus, with rate zonal ultracentrifugation it is possible to purify and concomitantly collect particles of a certain size and shape (Maskowicz, 1991). In this method, a step-wise gradient of buffers in different densities is built, and the sample is loaded in the bottom of the tube, with density set at the highest value. During centrifugation, the particles with a certain size travel through different densities forming distinctive layers (Maskowicz, 1991). The particles are separated by both their size and their ability to sediment (Anderson, 1966). The method produces a reproducible size distribution pattern of the particles, and therefore can be used as a method to analyze e.g., RNA or DNA (Lin et al., 2013), bacteria (Maskowicz, 1991) or proteins like different lipoproteins (Polacek, Byrne, and Scanu, 1988). Particles that are uniform in size, such as LDL-particles, tend to float in a narrow band (Polacek, Byrne, and Scanu, 1988). With rate zonal ultracentrifugation, it is possible to separate modified lipoproteins from native lipoproteins, as the lipolytically modified lipoproteins float in different manner comparerd to the native lipoproteins (Pentikäinen, Lehtonen, and Kovanen, 1996), although lipoproteins, size of which do not change in modifications, (e.g. LDL modified with elastase) float similarly as the native LDL (Polacek, Byrne, and Scanu, 1988). The largest particles are found in the top of the ultracentrifuge tube. In this thesis, rate zonal ultracentrifugation was used to separate freshly isolated extracellular lipid particles in groups of different size. From the different size groups, it was then possible to analyze several characteristics, such as lipid and protein compositions of the particles.

Materials

Biological material

Ethics statement

The use of human material conforms to the principles outlined in the Declaration of Helsinki. The studies were approved by the Ethics Committee of Helsinki University Central Hospital and the National Authrority for Medicolegal Affairs. All surgical patients (patients undergoing cardiac valve replacement, heart transplant operation, or endarterectomy operation) had signed an informed consent documents. Human plasma and buffy coat preparations were obtained from healthy blood donors, and they were by-products from the preparation of blood products for clinical use. Use of the for lipoprotein isolation and monocyte preparation was approved by the Finnish Red Cross Blood Service.

Clinical characteristics of the patients

Table IV Patients with aortic stenosis (Study II)

Age	Sex M/F	BMI	Diagnosis	Clinical history	Statin	Smoking	Dys-lipidemia	Valve leaflet weight (mg)
67	F	29	AS	DM	+	-	+	1892
82	F	22	AS+AI	HT, DM	-	-	+	633
73	M	23	AS	DM	+	+	+	605
70	F	26	AS	-	-	-	-	1773
81	M	24	AS	HT, KD	-	-	-	2067
74	M	N/A	AS	HT	+	-	+	930
58	M	28	AS	HT, DM, TIA/stroke	+	+	+	1876
75	F	30	AS	-	-	-	+	1476
80	M	22	AS	HT	+	+	+	1393
61	F	23	AS	-	-	+	-	1520
70	F	31	AS	TIA/stroke	+	-	+	659, 767
53	M	22	AS	-	+	+	+	1950, 1080
53	M	27	AS	HT	+	-	+	1827, 2367
64	M	37	AS	DM	-	-	+	1668
86	F	N/A	AS	-	-	-	N/A	852
72	F	21	AS	HT	+	-	+	779
43	M	37	AS	-	+	-	+	4367
37	M	27	AS+AI	HT	-	+	+	295
86	F	22	AS+AI	-	-	-	-	2308
75	F	30	AS	-	-	-	-	392
65	F	28	AS+AI	-	-	-	N/A	778
76	F	33	AS	HT, DM	+	-	+	1321
63	M	31	AS	HT	+	+	+	1594

AS=Aortic stenosis, AI=aortic insufficiency, DM=diabetes mellitus, HT=Hypertension, KD= kidney disease, TIA=transient ischemic attact,

Table V Control patienst with no aortic stenosis (Study II)

Age	Sex M/F	BMI	Diagnosis	Clinical history	Statin	Smoking	Dys-lipidemia	Valve leaflet weight (mg)
69	M	25	AI	HT	-	-	-	214
65	M	30	AI	KD, lung disease	-	-	-	290
79	F	27	AI	HT, DM, KD	-	-	-	173
62	F	25	AI	HT	-	-	-	135
71	M	21	AI	HT, KD	-	+	+	352
55	M	23	AI	HT	-	-	-	186
58	M	n/a	Dil CMP	KD	-	-	-	248
63	F	33	ICH	N/A	N/A	N/A	N/A	270
58	M	n/a	CHF	TIA/stroke, lung disease	+	+	-	413
42	M	27	SAH	N/A	N/A	N/A	N/A	314
58	F	59	PE	TIA/stroke	N/A	N/A	N/A	201
64	F	n/a	Dil CMP	-	-	-	-	291
74	M	26	AMI	N/A	-	N/A	N/A	436
66	F	22	ES	N/A	N/A	N/A	N/A	347

AI=aortic insufficiency, Dil CMP=dilated cardiomyopathy, ICH=intracerebral hematoma, CHF=congestive heart failure, SAH=subarachnoid hematoma, PE=pulmonary embolism, AMI=acute myocardial infarction, ES=epileptic seizure, N/A=data not available, AS=Aortic stenosis DM=diabetes mellitus, HT=Hypertension, KD= kidney disease, TIA=temporary ischemic attact

Table VI Coronary artery donors (Study I)

Age	Sex M/F	Diagnosis	Source of coronary artery	Coronary artery	Plaque class (Stary et al., 1994a; Stary et al., 1994b)	Area gradings
59	M	Dil CMP	Transplant	7LCX	I	1,2
49	M	CMP	Transplant	1RCA	I	1,2
				6RCA	IV	3,4
55	M	Cerebral contusion	Donor heart	4RCA	II	2
				1LCX	VI	3,4,5,6
44	F	SAH	Donor heart	7LCX	III	2,3
60	M	Heart failure	Transplant	6RCA	VI	3,4,5,6
35	F	SAH	Donor heart	6RCA	IV	3,4,5

Dil CMP = Dilated cardiomyopathy, SAH Subarachnoid hematoma, LCX = left circumflex artery, RCA = right coronary artery.

Carotid arteries

For the analysis, 302 pieces of carotid arteries was used. The samples were anonymous, and no clinical details were collected. All patients had undergone a planned endarterectomy operation to remove an atherosclerotic plaque from the carotid arteries.

Results and discussion

Accumulation and retention of lipid particles in the arterial intima is considered as the initiation of atherosclerosis (Williams and Tabas, 1995). The experiments presented in this thesis are aimed at describing the chemical and physical characteristics of the accumulated lipid particles and the pattern of lipid distribution in the coronary arteries, and comparing the lipid particles accumulating in aortic valves to the lipid particles in carotid arteries and to plasma lipoproteins. In addition to this, the possible effects that the extracellular lipid particles may have on development of atherosclerosis are described. In this study the data are combined from three different sites of arterial systems that are most susceptible to lipid accumulation: coronary arteries, aortic valves, and carotid arteries. Also the possible contribution of the extracellular particles on the inflammatory component of atherosclerosis and aortic stenosis was studied.

For this research, to describe the spatial distribution of lipids, 8 coronary arteries at different stages of atherosclerosis were studied. To further analyze the extracellular lipid particles in aortic stenosis and carotid artery atherosclerosis, 29 aortic valve leaflets and excised specimens in total from 302 pieces of carotid arteries were analyzed. Of the analyzed valves,15 were stenotic and 14 non-stenotic. From the coronary arteries, spatial distribution of lipids was analyzed with ToF-SIMS, an imaging mass spectrometry tool. The carotid artery intima was imaged with electron microscopy and electron tomography. The lipid particles isolated from aortic valves and carotid arteries were analysed with thin layer chromatography, lipid mass spectrometry, protein mass spectrometry, Western blotting, ELISA, rate zonal centrifugation, dynamic light scattering, circular dichroism.

Patterns of lipid accumulation in atherosclerosis of coronary arteries (I)

In this study, lipid accumulation in different stages of human coronary artery atherosclerosis was analyzed with time of flight secondary ion mass spectrometry (TOF-SIMS) using cryosections of eight coronary arteries. Stary describes atherosclerotic plaques as entities and classifies them according to the most affected area (Stary et al., 1994b). However, in this thesis, the plaques were divided into subareas and the classifications by American Heart Association and Stary were used as guidelines. The visual data derived from photomicrographs, however, does not extend to the chemical data other than histological stainings such as Oil Red O or filipin -stainings for neutral lipids or unesterified cholesterol (Kruth, 1984). For more detailed data of the distribution of lipids other than cholesterol or neutral lipids other methods, such as imaging mass spectrometry, must be used.

The distributions of fatty acids, different species of cholesterol, phospholipids, iron-ion, and vitamin E were determined with TOF-SIMS, and linked with stainings for apoB-100, glycophorin A, CD163-positive macrophages, and CD34/31 for neovessel formation of the sequential sections of the same plaques. The images generated from the data obtained from TOF-SIMS were also combined to view the spatial colocalization of different lipid classes. Finally, the presence of the various lipid classes was compared with that of variousproteins that had been visualized with immunohistochemistry.

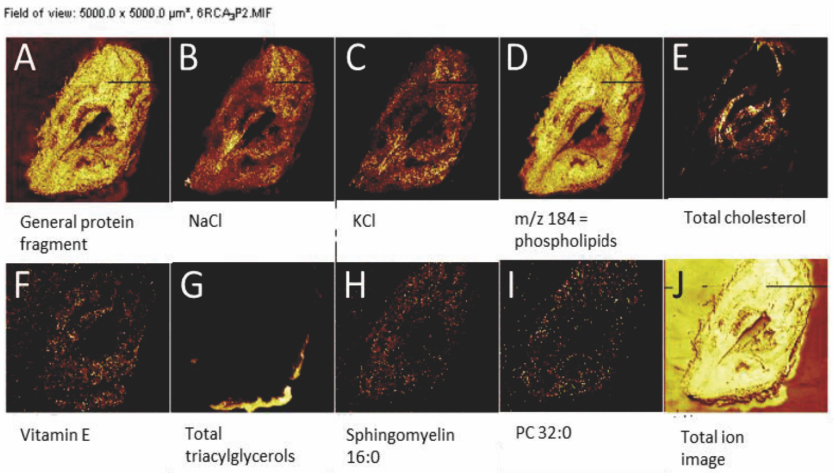

Figure 16 ToF-SIMS imaging mass spectrometry-generated images of small molecules and lipids detected on a coronary artery. Several different ions can be detected simultaneously, and both positive and negative mode ions can be detected. Large molecules, such as proteins, fragment and cannot be individually identified but small molecules of low molecular weight, such as lipids and vitamin E can be recognized. The spatial aggangement of different lipids, such as cholesterol, TAGs, and phospholipids can be compared with each other.

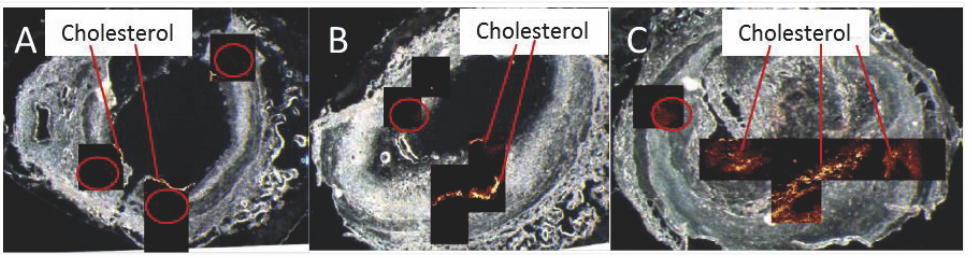

Figure 17. Cholesterol distribution in coronary arteries with a normal intima (A), and in an atheroma-stage (B), and an advanced stage atherosclerotic plaque (C). Three (A), six (B), and eight (C) areas of interest (500 x 500 µm) were analyzed in three atherosclerotic lesions with ToF-SIMS imaging mass spectrometry. Cholesterol is visualized by combination of all cholesterol ion species: data from ions m/z 385 and m/z 369 detected with a positive mode MS are combined and overlayed on the photomicrographs. A. In the normal coronary artery cholesterol signal can be seen mainly periendothelially. Deeper in the intima and in the media or adventitia the signal is much lower (circulated areas). B. In the atheroma-stage coronary artery the cholesterol signal seems to be accumulating deeper in the intima, close to the media. C. In a coronary artery with a very advanced atherosclerotic plaque, cholesterol is accumulated in several layers of a very thick intima

Imaging mass spectrometry (MS) revealed remarkable differences in spatial distribution patterns of the various lipids when thin sections of normal coronary arteries and of coronary arteries with different stages of atherosclerosis were compared (Figure 16). Imaging MS revealed that in an atherosclerotic intima, in one section of an artery, several different domains with different compositions of lipids can be detected. Next to the most affected area rich in cholesterol and phospholipids, there can be a domain that is more fibrotic or has less neovascularization and has less cholesterol or contains much less phospholipids. In the healthy coronary arteries examined, most of the lipids were unesterified cholesterol and phospholipids, lipid species common in cell membranes. In the later stages of atherosclerosis, cholesterol was seen to accumulate, first close to the internal elastic lamina, and later also closer to the lumen. Still, even in a very advanced plaque, part of the intima may resemble a normal intima with only very minor lipid depositions. Of the detected lipids, unesterified cholesterol and CE were directly correlated to the severity of lesions, as PC was inversely correlated with the advanced-type lesions. These features of human coronary arteries analyzed by TOF-SIMS will be discussed in the following chapters in more detail.

Cholesteryl esters and unesterified cholesterol

Cholesterol signals

Cholesterol was detected in several signals in TOF-SIMS. In the positive mode, m/z 385 (cholesterol - $H)^+$ and m/z 369 (cholesterol - $OH)^+$ were defined to represent unesterified cholesterol and esterified cholesterol, respectively. Using standardized samples of cholesterol and cholesteryl linoleate, the ratio of the two cholesterol species (m/z 385 and m/z 369 (ratio 385/369), interpreted as unesterified cholesterol and esterified cholesterol, respectively) was found to be 0.3 for unesterified cholesterol and less than 0.05 for esterified cholesterol (Study I, Figure 2). Thus, the lower the ratio, the more CE is detected. The ratio 385/369 is proposed to be a new marker for esterified cholesterol.

Cholesteryl esters and unesterified cholesterol in the coronary artery sections

In the normal coronary artery sections in this study, lipids were seen evenly distributed in the intima as well as in the media (Figure 17 A). Only near the endothelium cholesterol concentration was detected to be above the average of that sample. Based on the ratio 385/369, the cholesterol in the normal arteries was mainly unesterified cholesterol (m/z 385). The ratio 385/369 showed that the proportion of CE was the highest in the atheromas and in advanced stage lesions (Study I, Figure 2D). Unesterified cholesterol is found in the plasma membranes of all cells (Lange et al., 1989). The difference in the intensities of the different cholesterol species, m/z 385 and m/z 369, was substantial between normal intima and a fatty streak: the ratio 385/369 in fatty steaks (0.14) was half of the same ratio in the normal intima (0.3). In the atheroma-stage (0.07) and in the advanced lesions (0.1) the ratio 385/369 indicated a high concentration of CEs deep in the intima. Ratio 385/369 was one fourth of the ratio in the normal intima (Study I, Figure 1D). CEs can be a target of hydrolysis by LAL in the extracellular space lipoprotein-derived lipid particles (Hakala et al., 2003), thus affecting the ratio 385/369. These results show that in the fatty streaks, part of the cholesterol was now in esterified form (m/z 369), and as also apoB-100 was found in the same areas, CEs are likely to be derived from plasma apoB-100-containing lipoproteins, notably from LDL and possibly from VLDL-remants. In the atheroma stage, cholesterol m/z 369 was concentrated near the internal elastic lamina with linoleic acid, oleic acid, and palmitic acid, where alsoa dense distribution of vitamin E (m/z 430) was detected. These findings also support the presence of plasma lipoprotein-derived lipid particles. Vitamin E can be considered a marker of lipoproteins, as it travels as a component of them (Esterbauer et al., 1991) and it was found to co-localize with total cholesterol signal both in an atheroma coronary artery and in an artery with an advanced lesion.

Red blood cells as a source of unesterified cholesterol

In the atheroma-stage, no glycophorin A indicating red blood cells or iron deposits were found, while in the advanced lesions, glycophorin A-stainings indicated heavy deposits of red blood cells (Study I, Figure 6). Hyperplastic intima in the most stenotic arteries contained dense neovascularization (Study I, Figure 6), and red blood cells could leak through lesions in the vulnerable neovessels (Sluimer et al., 2009), and be a source of unesterified cholesterol in the intima (Virmani et al., 2005). That might explain the higher ratio of m/z 385 to m/z 369 in the advanced lesion than in the atheroma.

Phospholipids in the coronary artery sections

In normal coronary arteries, intima is a thin layer between endothelium and internal elastic lamina and only mild adaptive thickening can be noticed (Stary et al., 1992). The most common phospholipids are PC, LPC and SM that share a common choline head group ($C_5H_{15}NPO_4^+$). In the TOF-SIMS-analysis, the phospholipids were detected both as molecular ions either on positive ion mode (+) or negative ion mode (-): (PC 32:0, PC 34:3, PC 34:2, and PC 34:1; SM 18:1, SM 18:0, SM 16:1, and SM 16:0; LPC 16:0 and LPC 18:0 (Table 1, Study I), and ceramide. Total choline-phospholipids were detected as m/z 184, which was derived from the choline-headgroup. The intensities were normalized to the pixel count of the analyzed area.

Ratio of phospholipids to cholesterol

As the intensity of the common choline headgroup m/z 184 was determined by TOF-SIMS, the signal was found to be the lower the more advanced stage of atherosclerosis the analyzed areas were (Study I, Figure 2A). The lowest intensities were found in the advanced-stage lesions. Specific PC intensities were the lowest at the atheroma-stage and (Study I, Supplemental Figure 5A), as in the advanced-stage lesions the intensity was again 1.5-fold compared to the intensity in the atheroma. In the normal intima and in fatty streaks, the ratio of cholesterol to phospholipids (Chol/PL = m/z 385 + m/z 369 / PC + SM) was low (the value of the ratio is 1 in the normal intima, 2 in the fatty streak). In the atheroma, however, the ratio (11.1) was 15-fold higher than in the normal intima (0.7) due both to decrease in phospholipids and increase of CEs. In the advanced-stage artery, the ratio was lower than in atheroma (5.6), but still 8-fold higher than normal intima (Study I, Supplemental Figure 5). While the ratio was 2-fold compared to normal intima in the fatty streak-phase, it was not significantly higher although in the immunohistochemistry apoB-100 was already present. As in a normal intima with adaptive intimal thickening the most abundant cell population are smooth muscle cells (Nakashima et al., 2002), the dominance of phospholipids is consistent with the result of the measurements of Tulenko and co-workers, of smooth muscle cell membrane cholesterol/phospholipid ratio (0.96:1) (Tulenko et al., 1998). The Chol/PL-ratio in the coronaries with an advanced lesion was still high, but only half of the ratio observed in the atheroma-stage. Decline of total phospholipid ion m/z 184 intensity may derive from degradation of either PC or SM or both. Also intensity of SM affects to the Chol/PL-ratio, and SM-hydrolyzing SMase has been detected in atherosclerotic lesions (Marathe et al., 1999), and in accordance with that, a small increase of ceramide-intensities was detected in atheroma-stage lesions. Smith and her co-workers analyzed human aortae and extracted lipid from from whole plaques in different stages of atherosclerosis. In the fatty streak –phase the Chol/PL-ratio was 1.8—2.4 and in the atheroma-stage and in advanced lesions the ratio Chol/PL was 13.3.—15.5 (Smith, Slater, and Chu, 1968), which is well in accordance with the results acquired with TOF-SIMS.

Ratio of lysophosphatidylcholines to phosphatidylcholines

The highest intensity of LPC was found in the atheromas (Study I, Supplemental Figure 5 A). Also the ratio of LPC to PC (LPC/PC) was found to be different in different types of lesions in Study I. The ratio LPC/PC was the highest (0.9) in the atheroma-stage of coronary artery lesions. In the advanced-type lesions the ratio

of LPC/PC was lower (0.5) than in the atheroma-stage, but still higher than in the normal intima (0.2) (Study I, Figure 5). LPC may be derived from oxidation of PC (Esterbauer et al., 1992) or PC hydrolysis by PLA_2. Several PLA_2-enzymes have been detected in the atherosclerotic arteries, some of which can hydrolyze PCs in intact LDL, while some of them use modified LDL as substrate (Öörni and Kovanen, 2009), and also oxidation have been shown to lead to PC hydrolysis (Steinbrecher et al., 1984). Smith and co-workers extracted lipids from aortic lesions and the ratio LPC/PC was between 0.14—0.4 in fatty streaks and 0.5—0.9 in the necrotic core of advanced lesions (Smith, Slater, and Chu, 1968). Later LPC was found to be capable of *in vitro* activating macrophages into phagocytocing especially complement-bound targets, in this case red blood cells (Ngwenya and Yamamoto, 1985). PC hydrolysis products fatty acids and LPC are transported out from the plaque with albumin. LPC is capable in inducing secretion of inflammatory cytokines from human monocytes *in vitro*, and saturated LPC 18:0 and 16:0 had larger effect than unsaturated LPCs did (Liu-Wu, Hurt-Camejo, and Wiklund, 1998).

Fatty acids in the coronary artery sections

Fatty acids were detected in the negative mode of TOF-SIMS. The detected fatty acids were palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), and arachidonic acid (20:4). The fatty acids were partly free fatty acids, partly fragmented from CEs, TAGs, PCs, and SMs (Debois et al., 2009). In the normal coronary arteries, 16:0 and 18:0 were evenly distributed and found in all artery layers. 18:2 and 18:1 were found from the fatty streak to the advanced lesion-stage, where they co-localized with cholesterol. In LDL, the most abundant fatty acid is 18:2, which is esterified in CEs, and the second most abundant fatty acid is 16:0, in the phospholipids of LDL (Esterbauer et al., 1992) Oleic acid is abundant in the foam cells since ACAT, the enzyme that esterifies cholesterol into CEs in macrophage cytoplasm thus generating foam cells, prefers oleic acid (Brown, Ho, and Goldstein, 1980).

Triacylglycerols in the coronary artery sections

In the coronary arteries analyzed with TOF-SIMS, TAGs were detected as diacylglycerols (DAG). The molecular ion of TAG was very weak. Sjövall and co-workers showed in a principal component analysis that the signals of DAGs and TAGs have a strong positive correlation (Sjövall et al., 2008) and Debois and co-workers performed TOF-SIMS analysis on TAG species from which the result was strong signal of DAG-molecular ions (Debois et al., 2009). Based on these data DAGs were considered as a reliable marker of TAGs in this type of analysis. The detected DAGs in the Study I were 32:2, 32:1, 32:0, 34:2, 34:1, 36:3, and 36:2, and they were interpreted as TAGs according to the results of Sjövall and Dubois and their groups. A strong intensity of TAGs was found in the coronary samples with normal intima next to the endothelium. Interestingly, cholesterol and TAGs did not co-localize. In the advanced lesions, TAGs were located next to the endothelium of the coronary arteries at the sites of neovascularization, and also near to the endothelia of newly formed microvessels, while cholesterol was located deeper in the intima. This suggests either that the TAG-rich lipoproteins did not reach the site of cholesterol accumulation due to degradation of the TAGs in the tissue or due to steric hindrances, mainly because of the large size of the TG-rich particles. As the endothelium of atherosclerotic lesion sites is known to be eroded, i.e. local losses of endothelial cells (Mäyränpää et al., 2007), the larger lipoproteins can have acces from the coronary lumen to the coronary intima. The size distribution of particles that can enter the uncompromised endothelium was described by Nordestgaard and Zilversmit (Nordestgaard and Zilversmit, 1988): the entering particles are smaller than 75 nm, while particles larger than that do not cross the endothelium unless it is leaky (McDonald, Thurston, and Baluk, 1999). Another origin of TAG in the intima may be the foam cells. In vitro-experiments by Boström and co-workers show, that if macrophages are exposed to hypoxia, the lipid droplets of these macrophages are enriched with TAGs (Boström et al., 2006).

Lipids in the media

In the sections of media that were next to intima with atheroma-stage or advanced lesion, higher lipid ion intensities were detected than in the media sections next to normal intima. Both total cholesterol and vitamin E -intensities had significantly higher normalized intensities in the media next to atherosclerotic intima than in the media next to normal intima, and this suggests increased infiltration of plasma lipoproteins into the mediaIn the areas of media that were next to advanced plaque areas, the media contained more total cholesterol, and vitamin E, and also more PCs than in the areas of media that were next to a healthy intimal areas (Study I, Supplemental Figure 7). Previously, media lipids have seldom been analyzed in atherosclerosis. In 1966, Insull and Bartsch extended their analysis also to the media of thoracic aorta, and saw "occasional finely dispersed lipid adjacent to the intimal fatty streak" (Insull and Bartsch, 1966). They report that patients with atherosclerotic lesions had slightly higher lipid content in the media compared to non-atherosclerotic patients, but the difference was not statistically significanat. However, they found slight correlation between medial cholesterol content to the fatty streak cholesterol content (Insull and Bartsch, 1966). Thus the lipids in the intima seem to affect slightly the lipid composition of the media. This may be due to elastolytic fragmentation of internal elastic lamina in the plaque as the macrophages in the intima secrete MMPs (Pasterkamp et al., 2000). Smith and Staples found earlier that when internal elastic lamina was intact, LDL concentration on the medial side of internal elastic lamina was only 0.3 % of that in the intima, and if the internal elastic lamina was damaged, medial concentration of LDL increased significantly (Smith and Staples, 1980). Also Sinapius report that large atheromas are associated with medial lipid (Sinapius, 1980). Evidence that point to this direction were found also in this study. As atherosclerosis is considered as an intimal disease, these findings were interesting, but due to small sample size, more evidence is necessary before definitive conclusions can be drawn.

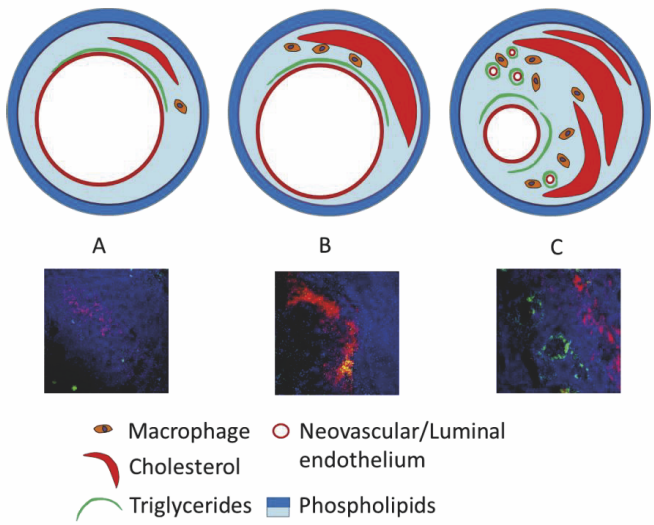

Figure 18 Spatial distribution of lipids in a normal, (A), atheroma-stage (B), and an advanced-stage (C) coronary artery. Schematic drawing and a ToF-SIMS-generated image of respective stages of atherolsclerosis. cholesterol and triglycerides co-localize only minimally in the atherosclerotic intima. Cholsterol = red, Triacylglycerols = green, phospholipids = blue.

While the MS-imaging analysis of coronary artery sections shows the total lipid burden of a certain area of an artery (Study I), several signs of lipid modifications could be detected. The Chol/PL-ratio, where the ratio changes between the atheroma-stage and advanced lesion-stage reflected changes not only in cholesterol levels but also in levels of PC and SM, and LPC/PC-ratio, which showed the degree of PC degradation, were

both significantly different in the atheroma-stage plaques of a coronary arteries compared to a normal coronary artery intima.

Previously, no detailed analysis of spatial lipid composition of the human coronary arteries has been performed. In this study, TOF-SIMS analysis of the coronary arteries revealed that the lipids form distinctive domains in the artery during atherogenesis (Figure 18) and extend even to the media. Cholesterol species, especially esterified cholesterol, were accumulated deep in the intima, close to internal elastic lamina. The cholesterol seems to be derived from lipoproteins in the early lesions while in the advanced lesions, the cholesterol seemed to be derived from both lipoproteins and red blood cells, TAGs seemed to accumulate separately from cholesterol and were found periendothelially both near the neovessels and the arterial lumen. Possible sources of TAGs are chylomicron remnants, VLDL and IDL-particles. In the analysis of spatial distribution of lipids in different stages of atherosclerosis, it was also possible to see detailed spatial information of the native and modified lipids in the intima.

Particle modifications in arterial intima and in aortic valve (II and III)

With imaging MS it was possible to show microscopic and detailed information of spatial distribution and the composition of the lipids that had accumulated in the arterial intima at different stages of atherosclerosis (Figure 18). However, TOF-SIMS signal contained all lipids acquired from the tissue section surface, both from the cell membranes and the extracellular lipid particles. Thus distinguishing the source of the lipids, that is, whether the lipids originated from plasma lipoproteins, or if they were derived from dead foam cells and were of intracellular origin, was not possible with TOF-SIMS. Also, In study I, comparison of immunohistochemistry and TOF-SIMS showed, that esterified cholesterol and apoB-100 were found in the same areas of the atherosclerotic plaques. These results gave a rationale for isolating the extracellular lipid particles and further analyzing the particles from sites prone to atherosclerosis in Studies II and III.

The extracellular lipid particles were isolated from human aortic valve leaflets (Study II) and human carotid arteries (Study III). The isolated lipid particles were analyzed for their lipid composition, oxidation status, and size. The particles isolated from aortic valve leaflets and carotid arteries resembled the particles found in works by Guyton and co-workers (Guyton and Klemp, 1994), Hoff and co-workers (Hoff and Gaubatz, 1982; Hoff and Morton, 1985), and Chao and co-workers (Chao et al., 1988; Chao et al., 1990). Lipid analysis by thin layer chromatography and mass spectrometry showed that the lipid contents of the particles were different from plasma LDL and VLDL, and the particles in the stenotic aortic valves showed different lipid composition from the particles isolated from the coronary artery plaques. The proportion of PC was decreased both in the particles isolated from aortic valves and in the particles isolated from carotid arteries, and the proportion of free fatty acids was increased in the particles isolated from either tissue when compared to the plasma LDL lipid composition. The lipid particles showed signs of modifications by oxidation and diverse lipolytic and proteolytic enzymes. The physical and chemical characteristics of the extracellular lipid particles will be discussed in more detail in the following chapters.

Sizes and rate zonal floating of the extracellular lipid particles

The extracellular lipid particles isolated from stenotic aortic valves (Study II) and carotid artery lesions (Study III) had common properties. Particles isolated from both sources were larger than plasma lipoproteins, but mainly smaller than the intracellular lipid droplets, the size of which can be even several micrometers (Figure 19). Dynamic light scattering shows that the extracellular lipid particles in aortic valves (Study II) had an average diameter of 400 nm (range 30—1000 nm) and the average diameter of the particles isolated from carotid arteries was 500 nm (range 40—1000 nm). The size-range of the experimental intracellular lipid particles (Study II) overlapped the size-range of the particles isolated from carotid arteries

(Figure 19; Study III, Figure 3). Dynamic light scattering does not separate large spherical particles from large particle aggregates. Thus part of the largest particles may also be aggregates formed of several smaller particles or particles in process of fusing together.

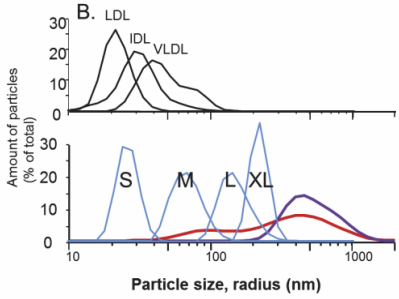

Figure 19. Sizes of the plasma lipoproteins and the extracellular lipid particles. The sizes of plasma lipoproteins (top panel) and extracellular lipid particles from aortic valves and carotid artery plaques (lower panel, blue and red, respectively) were measured with dynamic light scattering. The extracellular particles were in average larger than plasma lipoproteins. Also experimental intracellular lipid droplets were analyzed (lower panel, in purple).

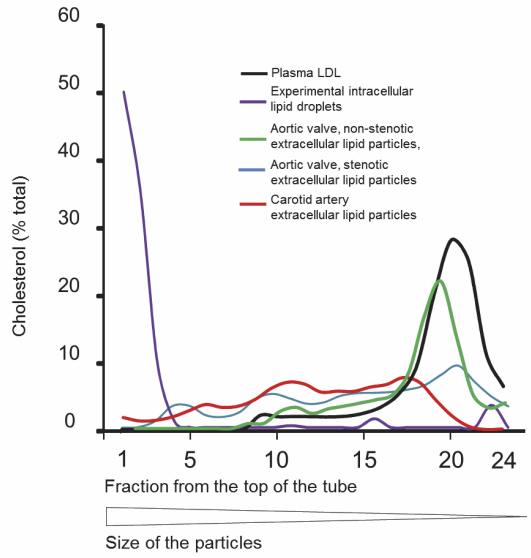

Figure 20. Rate zonal analysis profiles of extracellular lipid particles,experimental lipid ddroplets, and LDL. Several rate zonal ultracentrifugation analysis were combined in the figure: experimental intracellular lipid droplets (orange), extracellular particles isolated from healthy aortic valve (green), stenotic aortic valve (blue), and carotid artery plaques (red), and plasma LDL (black line). The experimental intracellular lipid droplets floated in a top layer of the tube, while LDL-particles floated near the bottom ot the tube. The extracellular lipid particles from healthy aortic valves floated quite similarly to the LDL-particles. The extracellular particles from stenotic aortic valves and from the carotid artery plaques are distributed throughout the layers.

When the aortic valve and carotid artery plaque extracellular lipid particles were separated by rate zonal ultracentrifugation, four distinct size-peaks in the flotation profiles were observed (Figure 20, blue line). After measuring the peak fractions with dynamic light scattering, the groups were termed according the sizes of the particles XL, L, M, and S, where the S-sized particles were close to the size of LDL and XL-sized particles were close to the size of experimental intracellular lipid droplets (Figure 19; Study II, Figure 3). The particles that were isolated from the healthy aortic valves (Figure 20, green line) floated very close to the LDL particles (Figure 20, black line). The extracellular lipid particles isolated from the carotid artery plaques, however, were distributed troughout the layers, as the particle size variation was large. Most of the apoB-100, as measured by ELISA, was found in the smallest particles that were of the same size as LDL both in the aortic valves and in carotid artery plaques (Figure 21; Study II, Figure 3B-C, Study III, Figure 3B).

With rate zonal ultracentrifugation analysis, it could be seen that the size of the particles were very different from those of the plasma lipoproteins, but they also differed from the intracellular lipid droplets. Enzymatic modifications change the way how the lipid particles float in the ultracentrifugation. Pentikäinen and co-workers modified native LDL with several different methods (e.g. by proteolysis, Cu^{2+}-oxidation, SMase-lipolysis, and glycosylation), and the particles that had been proteolyzed, Cu^{2+}-oxidized, bound to LDL-antibody complexes, or lipolyzed, were found to float throughout the layers of rate zonal analysis (Pentikäinen, Lehtonen, and Kovanen, 1996). In Studies II and III, the rate zonal analysis profiles of the extracellular lipid particles were similar to those in Pentikäinen's work, which suggested that high degree of modification of the plasma lipoproteins from which the particles were derived had occurred in the tissues.

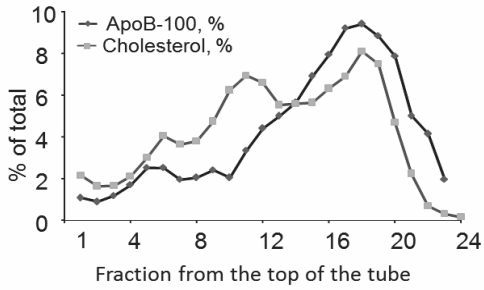

Figure 21. Cholesterol and apoB-100 in the carotic artery extracellular particles analyzed with rate zonal ultracentrifugation. Fractions collected from ultracentrifugation tube were analyzed by ELISA-for apoB-100-content and fluorometric Amplex Red analysis for cholesterol content. The analyses of the particles showed that while cholesterol was found in all fractions, intact apoB-100 was found mainly in the particles that floated in same layer as native LDL. Figure shows average results of two samples. Particles that floated on the surface of the tube were larger than the particles that were collected from the bottom fractions of the tube.

Lipid analyses

As the lipid particles isolated from the carotid arteries were isolated from pools consisting of several carotid artery samples derived from several different patients, and the particles were not fractioned by size, each value obtained represented an average of all particles in the particular pool (Study III). In contrast to that, the particles from aortic valves derived from individual samples (one valve leaflet at a time). The particles isolated from a single valve leaflets were fractionated by size, and this enabled studies yielding information from individual patients (Study II). The possibility to collect data from an individual stenotic valve leaflet, rather than from a pool of several atherosclerotic carotic arteries, revealed large variation in the lipid particle lipid compositions not only between the the different sized particles but also between individuals (Figure 22) In the samples derived from some patients the small particles resembled LDL in size and lipid composition, whereas in other samples the particles were more VLDL-like with large amounts of TAGs and with lower amounts of CEs. In the pools of carotid extracellular particles, which represented averages of all patients in the particular isolation pool, the lipid composition of the particles had features from LDL rather than from VLDL, as the proportion of CEs was much higher than the proportion of TAGs. Next, these modifications in lipid compositions will be discussed.

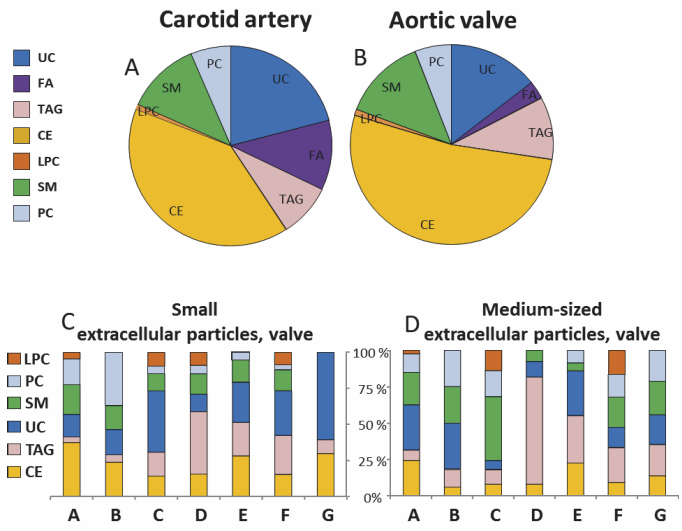

Figure 22. Lipid contents of the extracellular lipid particles. Lipid contents were analyzed from extracellular particles isolated from stenotic aortic valve leaflets (A, C, and D), and carotid artery plaques (B). In the pie charts, several pieces of aortic valve or carotid artery were combined for analysis (A, B). In the columns (C, D), each column represents lipid particles isolated from an individual patient. Lipid analysis was performed from particles that were sorted by their size. LPC = lysophosphatidylcholines, PC = phosphatidylcholine, SM = sphingomyelin, UC = unesterified cholesterol, TAG = triacylglycerol, CE = cholesteryl ester Columns A-G denote different patients.

Phospholipids and effects of phospholipolysis in the extracellular lipid particles

Phospholipids constitute the primary portion of the surface monolayer of the lipoproteins and the bilayer of the cell plasma membranes. The most abundant phospholipid in LDL is PC, which comprises 14 % of total lipids. Of LDL phospholipids, 64 % is PC followed by SM and LPC, with 26 % and 11 % contributions, respectively (Esterbauer et al., 1992). Phospholipolysis of these lipids can produce ceramides, DAGs, lysophospholipids, and free fatty acids, which are also found in LDL particles in minute amounts. LDL particles also contain small amounts of PE, PS, and PI.

Phosphatidylcholine and lysophosphatidylcholines

In LDL, the main phospholipid is PC (Figure 23), of which the most abundant PC is PC 34:2, with fatty acyl chains 16:0 and 18:2. The extracellular particles isolated from stenotic aortic valves and carotid arteries shared common features: In aortic valve extracellular particles and in the carotid plaque extracellular particles, the most common PC was PC34:1 (16:0/18:1) (Study III, Supplemental Figure IV). Interestingly, the aortic valve and the carotid artery extracellular lipid particles both were found to contain several PC-species that may contain arachidonic acid in their sn2-position, such as PCs 36:4 (16:0/20:4), 36:5 (16:1/20:4), 38:4 (18:0/20:4), and 38:5 (18:1/20:4), and the proportions of 36:4 and 38:4 were almost 9 mol-% of the total PC. Arachidonic acid of membrane PCs is an upstream molecule in the cyclo-oxygenase and leukotriene pathways, and, as such it is a source of prostaglandins and thromboxanes, and leukotrienes, respectively (Samuelsson, 1991). Thus, arachidonic acid can be considered a potentially proinflammatory molecule (Calder, 2006). In feeding experiments of piglets, dietary arachidonic acid has been found to

become incorporated into LDL phospholipids, when the arachidonic acid in their feed was introduced in TAGs (Amate, Gil, and Ramirez, 2001).

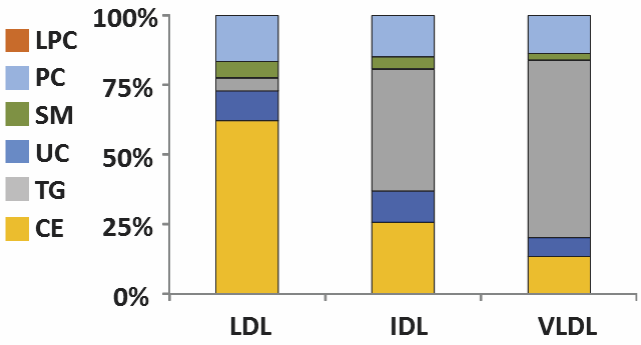

Figure 23. Lipid compositions of plasma lipoproteins LDL, IDL, and VLDL. LDL, IDL, and VLDL lipid compositions were analyzed by TLC. © Lehti et al (Lehti, 2013)

The total proportion of PC in lipid particles isolated from either aortic valves or carotid arteries was lower than it was in LDL The lipid contents of all extracellular lipid particles were different from both LDL and VLDL. PC/SM-ratio in the particles was lower than in LDL, due to the lower of PC-content of the particles. A large variation in aortic valve lipid profiles between individual patients was found. The proportion of PC in small aortic valve lipid particles varied from 40 % (patient B, Figure 22) to 0 % (patient G) The low PC/SM-ratio likely indicates the loss of PC, and apart from oxidation that will be introduced later, a possible source for the LPC and free fatty acids could be hydrolysis of PC with PLA_2-enzymes. PLA_2-enzymes are phospholipases that are able to hydrolyze the ester bond between a sn2-carbon of a glycerol backbone and an acyl chain in a phospholipid. More than thirty PLA_2-enzymes have been already described (Murakami et al., 2015), and 10 of them are recognized in the atherosclerotic intima (Öörni and Kovanen, 2009). PLA_2-IIa that uses oxidized LDL as its substrate, has been found in the valve tissue (Kupreishvili et al., 2011), and high concentrations of Lp-PLA_2 are found in plasma of patients with severe aortic stenosis (Kolasa-Trela Renata, 2012), and in Study III, PLA_2-V was seen in the carotid artery lesion in the immunohistochemistry, albeit in modest intensity (Figure 24). Lp-PLA_2 has also been associated with carotid artery atherosclerosis (Goncalves et al., 2012; Mannheim et al., 2008), and its highest expression is found in the necrotic core of a plaque. Hydrolysis of PC is a prominent phenomenon occurring in the extracellular lipid pool and can affect the aggregation of the lipid particles and hence the uptake by macrophages (Griffin et al., 2005). Also fusion of LDL-particles by the action of PLA_2-hydrolysis has been shown (Hakala et al., 2001). The physical properties of isolated particles in Studies II and III point to the direction that PLA_2-hydrolysisis is partly responsible of such modifications.

In coronary arteries in Study I, LPC was found to be enriched in atheroma and in complicated lesions. However, in the extracellular lipid particles that were isolated from aortic valves (Study II) or carotid arteries (Study III), the proportion of LPC (0.8 %) in atherosclerotic carotid artery lesions was, surprisingly, not found more pronounced than in plasma LDL (2.7 % (Esterbauer et al., 1992)), although the proportion of PC was less than half (6.4 %) of that in plasma LDL (14.5 %, (Esterbauer et al., 1992)). Also, somewhat surprisingly, the proportion of LPC in the aortic valve leaflets (2.8 %) was not significantly higher than in LDL (Figure 22 A-B). These low levels of LPC were found in pools of several carotid artery plaques or aortic valves. The seemingly low levels or even apparent loss of LPC was hypothesized to be derived from the analysis method. An averaging effect was a result of an act of pooling the lipid particles that had been isolated from several valve leaflets or pieces of carotid artery plaques. As Figure 22 panels C and D showed, particles isolated from individual patients had very different proportions of LPC in the lipid compositions.

The lipid particle from several patients contained little or no LPC while the particles from some patients were rich in LPC. When the individual samples were combined as a pool, the extrema values were not detected. Another possible reason for low LPC-levels in the extracellular particles could be albumin, which can transport not only fatty acids but also LPC (Switzer and Eder, 1965). When the valve leaflets were viewed individually (patients A-G in Figure22), and the lipid particles were separated by their sizes from each valve leaflet, the proportion of LPC was found to vary between individuals (Study II, Figure 7A). LPC was found especially in the small lipid particles that closely resembled LDL in size and also in the medium-sized particles that were close to VLDL in their size. In some aortic valve samples, PC seemed to be non-hydrolyzed, and PC/SM-ratio resembled that of LDL, while in some samples, PC/SM-ratio was lower than in LDL and VLDL, where the ratios were found to be 2.9 and 3.3, respectively (Study II), consistent with Esterbauer and co-workers (Esterbauer et al., 1992). In the small aortic valve lipid particles, the ratio varied between 2.2 and 0.2 (Study II), and in the carotid arteries combined, the ratio was 0.5 (Study III). In the whole tissue extracts by Smith and co-workers PC/SM-ratio was 1.05 in the fatty streaks and in the necrotic core it was 0.11, as large amount of PC seemed to have been degraded (Smith, Slater, and Chu, 1968), however, there the proportion of LPC was again surprisingly low. Chao and co-workers (Chao et al., 1990) isolated lipid particles from human atherosclerotic abdominal aortae. They classified the particles as "esterified cholesterol-rich lipid particles" ($d < 1.01$ g/ml) and "unesterified cholesterol-rich lipid particles ($d = 1.03$—1.05 g/ml), The unesterified cholesterol-rich lipid particles were multilamellar vesicles (Chao et al., 1990). The PC/SM ratios were 0.6 and 0.5, respectively, similar to the carotid extracellular lipid particles in Study III.

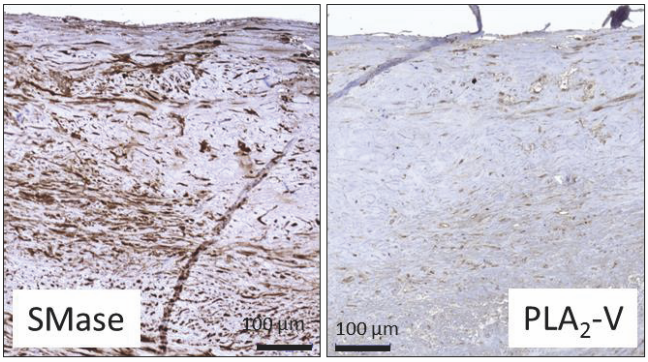

Figure 24. Sphingomyelinase (SMase) and phospholipase A_2-V (PLA_2-V) in carotid artery intima. Immunohistochemical analysis of SMase and PLA_2 in a carotid artery intima. SMase and PLA_2 are secreted by the endothelial cells and intimal macrophages in the atherosclerotic intima.

Sphingomyelin and ceramide

SM is the second-most abundant phospholipid in LDL and also in the extracellular lipid particles that were isolated from aortic valves and carotid artery lesions. SM can get hydrolysed by (acid) SMase to produce ceramide and a phosphocholine. As SMase is detected in the arterial intima (Marathe et al., 1999; Marathe et al., 1998) (and it was also detected in the carotid artery intima in this thesis (Figure 24), ceramides were expected to be detected in the lipid particles that were isolated from atherosclerotic carotid artery lesions. Unexpectedly, no ceramide was found in the isolated extracellular particles, while in the Study I, ceramides were detected in the atheroma-stage coronary artery sections in low intensities. Also the extracellular particles isolated from the aortic valve leaflets were devoid of ceramides. In the coronary atheromas, ceramides were found to have a slightly higher intensity compared to a normal intima; however, the intensities of SMs were ten-fold compared to the intensities of ceramides (Study I). Schissel and co-workers (Schissel et al., 1998; Schissel et al., 1996) isolated extracellular lipid particles from human atherosclerotic plaques from aortic aneurysms, and found that aggregated lipid particles were enriched in ceramide, while

the unaggregated particles were not. In the aggregated particles they found 10-50-fold increase of ceramide compared to plasma LDL. The differences between the present results and those obtained by Schissel and co-workers may be rooted to the different methods of isolation of the lipid particles and the detection method used in our study. They isolated the aortic lipid particles using an affinity immunoabsorption column with a polyclonal apoB-100-antibody, which excluded all but apoB-containing particles (Rapp et al., 1994). In their papers, Schissel and co-workers used DAG kinase using [y-32P]ATP to phosphorylate ceramide and DAG (Schneider and Kennedy, 1976) from the samples in purpose to analyze the amount of ceramide in the samples. In the current works (Study II and III), the extracellular lipid particles were not fractionated according their protein content, and mass spectrometry was used to analyze ceramide from the particles. Ceramide has two hydrophobic acyl tails and a neutral head group, and has thus a limited ability to become ionized. As the mass spectra were recorded from a mixture of lipids, the competition of ionization favoured the phospholipids that were more easily ionized, i.e. PCs and SMs. Thus mass spectrometry is likely to record ceramide well only if the proportion of it in the lipid mixture is extremely high or if ceramides are isolated from the total lipid mixture and measure separately.

Cholesterol and neutral lipids in the extracellular lipid particles

CEs are the most abundant neutral lipids in the core of LDL-particles, whereas TAGs are the most abundant neutral lipids in the the core of VLDL. The lipoprotein core can be hydrolyzed in macrophages by LAL that hydrolyzes the ester bond of CE to produce unesterified cholesterol and free fatty acids and TAGs into glycerol and free fatty acids. LAL can also be found pericellularly and has been shown extracellulary in human atherosclerotic arterial intima (Hakala et al., 2003).

Cholesterol and cholesteryl esters

Imaging MS of the coronaries showed that cholesterol distribution in plaques of different stages of atherosclerosis followed the distributions of apoB-100 and vitamin E (Study I). As vitamin E is carried in lipoproteins (Esterbauer et al., 1991) it suggested that the cholesterol in the coronary arteries was at least partly derived from plasma lipoproteins (Study I). Extracellular lipid particles isolated from both the aortic valves (Study II) and from the carotid arteries (Study III) contained large amounts of unesterified cholesterol (Figure 22). CE is the most abundant lipid species in LDL, and also in the extracellular lipid particles of carotid arteries and in the total lipid pool of aortic valve leaflets. In the lipid particles isolated from aortic valve leaflets and which had been fractionated according to their size, CEs were most often found in the particles that contained the highest concentration of apoB-100 as analyzed by ELISA (Study II). Unesterified cholesterol was found in the larger particles that also contained much less antibody-recognized apoB-100 and/or apoE. CEs of the intimal extracellular lipid particles contain the same type of fatty acids as do plasma lipoproteins, but in very different proportions. Cholesteryl linoleate (CE 18:2) is the most abundant CE in LDL and it was also most abundant in the extracellular lipid particles both from the aortic valve leaflets and from carotid artery lesions. Interestingly, in the aortic valve lipid particles CE 18:3 was the second most abundant CE, although in LDL and in the carotid lesion lipid particles the second most abundant CE was cholesteryl oleate (CE 18:1). The third most abundant CE in the carotid extracellular lipid particles was CE 18:3 that contained either the essential fatty acid α-linolenic acid, (ALA;18:3(n-3)) or gamma-linolenic acid (GLA; 18:3(n-6)). ALA is derived only from diet but GLA can be produced endogenously from linoleic acid 18:2. Mass spectrometry used in this analysis cannot distinguish between the two species, as it only detects the mass-charge relationship of the molecules.

The extracellular lipid particles isolated from the carotid arteries in Study III contained particles that were the same size as the experimental intracellular lipid particles (Figure 19, purple line). As mentioned above, the most abundant CE in LDLis CE 18:2 (Liu, Bagdade, and Subbaiah, 1995). In the foam cells, however,

CE 18:1 is the most abundant CE. In the cells, the LDL CEs are hydrolyzed in the lysosomes into unesterified cholesterol and an acyl chain, and the unesterified cholesterol is re-esterified by aceyl coA:cholesteryl acyltransferase / Sterol O-acyltransferase 1 (ACAT/SOAT1) to be stored in foam cell lipid droplets (Brown, Ho, and Goldstein, 1980). ACAT/SOAT1 prefers oleic acid as a substrate over linoleic acid, and thus the main CE in the intracellular lipid droplets is cholesteryl oleate. The ratio of cholesteryl oleate to the sum of cholesteryl oleate and cholesteryl linoleate (18:1/(18:1+18:2)) in the carotid artery lipid particles in Study III was 0.35, and in LDL it was 0.18. In stenotic aortic valves of Study II the ratio was 0.19. According to Guyton and Klemp (Guyton and Klemp, 1994), this ratio in the extracellular lipid particles is between 0.2 and 0.47, while in intracellular lipid droplets it could be as high as 0.8. That suggests that the extracellular lipid pool was mostly of lipoprotein origin, but that it may also contain small amounts of intracellular lipid droplets derived from apoptotic foam cells that had released their lipid droplet contents into the extracellular compartment. In Study I linoleic, oleic and palmitic acids were found to co-localize with cholesterol m/z 369 in the coronary artery sections, mainly in the atheroma- and advanced plaque -stages of atherosclerosis, in the TOF-SIMS-generated images (Study I, Figure 3), and thus be CE-derived.

An interesting group of CEs in the carotid extracellular lipid particles were the CEs with odd number of carbon acyl chains, CE 19:2, 19:1, 17:0, 17:1, and 15:0. The combined proportion of these species was low in LDL, 2.6 % of measured CEs, but in extracellular lipid particles the proportion was higher, 11.3 %. In the carotid particles CE 15:0 was found to have higher proportion than CE 17:0. Synthesis of 15- and 17-carbon chains has been described already in the 1960's in rat adipose tissue (Horning et al., 1961). Contemporarily, they have been found to be synthesized also in human tissues: red blood cells, human breast milk, and adipose tissue contain saturated odd-chain fatty acids, and fatty acid 17:0 generally dominates over fatty acid 15:0, as reviewed by Jenkins and co-workers (Jenkins, West, and Koulman, 2015). Another source of these lipids can be cow's milk (Baumann et al., 2016; German and Dillard, 2006), and the odd number carbon acyl chains can also be derived from bovine meat, mutton, and pork (Mitchaothai et al., 2007), but can also be derived from plant sources. In dairy fat, the fatty acid 15:0 is the dominant one over fatty acid 17:0 (Jenkins, West, and Koulman, 2015). That suggests that the fatty acyl moiety of thee odd-chain CEs in the extracellular lipid particles would be at least partly derived from a dietary source. The β-oxidation product of these fatty acids, propionyl-CoA, can be utilized as a substrate in the citric acid cycle in all cells, and the odd-chain fatty acids are thus catabolized efficiently. Also, the intracellular enzyme LAL can use CE of various chain lengths as a substrate (Sheriff, Du, and Grabowski, 1995). Thus the reason for their accumulation is not clear.

Triacylgylcerols and free fatty acids

TAGs are the main lipid species of VLDL and there are still minor amounts of them in the plasma LDL (Figure 23). In the extracellular lipid particles of aortic valve leaflets (Study II) and in the carotid arteries (Study III), the proportion of TAGs was very similar. Similarly with the phospholipids or CEs also the TAG-content of the extracellular lipid particles isolated from extracellular lipid particles from aortic valves had a high individual variability between patients. Some patients had LDL-like TAG-content in the stenotic aortic valve particles, while the particles isolated from other patienst resembled more VLDL by their lipid composition (Figure 23).

LPL is found on the luminal side of capillary endothelium, and it is possible that the TAG-rich particles are stripped from the TAG load before they even enter the intima (Pentikäinen et al., 2002). Small VLDL-remnants can have an access into the intima (Nordestgaard and Zilversmit, 1988), and TAG-rich particles have an access into the intima through a damaged endothelium (Frank and Fogelman, 1989). Free fatty acids are used as energy source for the cells in the arterial wall and for the heart muscle, and they are produced from TAGs by LPL on the endothelial cells for that purpose. LPL mRNA is expressed by macrophages, but

only in modest quantities (Mattsson et al., 1993). Phospholipids are also a source of free fatty acids, and especially arachidonic acid is carried in phospholipids to be used as a material for prostaglandin and leukotriene synthesis. The isolated extracellular particles from the aortic valves and the carotid arteries contained free fatty acids in quantities that are not found in circulating plasma lipoproteins (Study II and III). Lähdesmäki and co-workers have presented data showing that acidic pH tends to prevent the products of lipolysis from leaving the LDL-particles (Lähdesmäki et al., 2009). Here, the free fatty acids source was likely the PC, the proportion of which was dramatically decreased when compared to plasma lipoproteins. Also the CEs may have been a source of free fatty acids, as LAL, that has been shown to exist also pericellularly (Hakala et al., 2003), cleaves the fatty acids from the lipoprotein core producing unesterified cholesterol and free fatty acids. Free fatty acids can be pro-inflammatory (Dasu and Jialal, 2011; Joosten et al., 2010), and the free fatty acids are also considered cytotoxic when their production exeeds their demand (Jaishy and Abel, 2016; Schaffer, 2003).

Oxidation in the extracellular lipid particles

Malondialdehyde (MDA) and malondialdehyde-acetaldehyde (MAA) adducts are products of lipid peroxidation found in phospholipids and lysine residues of proteins, and thus also epitopes in oxidized LDL are found in atherosclerotic lesions (Hill et al., 1998; Rosenfeld et al., 1990). Both the aortic valve extracellular particles and carotid artery extracellular particles were analyzed the same way for signs of oxidation: the apoB-100 lipoprotein-containing particles were tested for their antigenicity against MDA and MAA. For the analysis, four antibodies generated against MDA-LDL and MAA-LDL epitopes (Turunen et al., 2012; Veneskoski et al., 2011) were used. The probed antibodies revealed the presence of oxidized epitopes in the extracellular lipid particle isolate both from the aortic valves (Study II) and from carotid arteries (Study III). In plasma LDL samples that were probed alongside with the samples of either aortic valve lipid particles or carotid artery lipid particles contained only minimal amounts the epitopes. The extracellular lipid particles were isolated in the presence of EDTA, which is suggested to prevent the *ex vivo* –oxidation (Steinbrecher et al., 1984), and thus the detected markers of oxidation had already been present before the isolation process.

The extracellular lipid particles isolated from human aortic valve leaflets (Study II) and human carotid arteries (Study III) showed signs of modifications that suggest also possibility of oxidation of the lipid particles, such as high concentrations of LPC and high amounts of unesterified (= free) fatty acids. These features (increase of free fatty acids and change of density) can be results of phospholipase hydrolysis, but is also similar to the results observed by Steinbrecher and co-workers after oxidation of LDL (Steinbrecher et al., 1984). Smoking is an exogenous factor that can oxidize LDL and thus affect atherosclerosis and aortic stenosis (Syväranta et al., 2014; Yamaguchi et al., 2001).

Cholesterol crystals

Previously, Guarino (Guarino, Tulenko, and Wrenn, 2004) showed, that by first hydrolyzing LDL with CEase and then subjecting the lipolyzed LDL to hydrolysis by SMase, cholesterol crystals appeared in the solution. Based on this background knowledge, in Study III a different approach was used by following the works of Chao (Chao et al., 1992) and Plihtari (Plihtari et al., 2010). Thus, the surface of the LDL was first destabilized by oxidation, lipolysis, or proteolysis, and thereafter the core CEs were hydrolyzed with LAL. After such double-modification, cholesterol crystals were found to be generated in vitro from LDL and VLDL (Figure 25; Study III, Supplemental Figure I). When the lipoprotein particles were pretreated with lipolysis, proteolysis, or oxidation to destabilize the surface monolayer of the lipoproteins, cholesteryl esterase was able to get in contact with CEs and hydrolyze them. This produced unesterified cholesterol, which is the prerequisite of cholesterol crystal formation. Such crystals were seen in ToF-SIMS-generated

images (Figure 26; Study I, Figure 7). Cholesterol crystals were also seen in TEM electron microscopy (Figure 27; Study III, Figure 1) and 3D-electron microscopy images of atheroma in carotid arteries (Figure 28; Study III, Figure 1).

Figure 25. An *in vitro*-generated cholesterol crystal. LDL was hydrolyzed with SMase and LAL and incubated in +37 °C for 48 h. Formed crystals were imaged under polarized light.

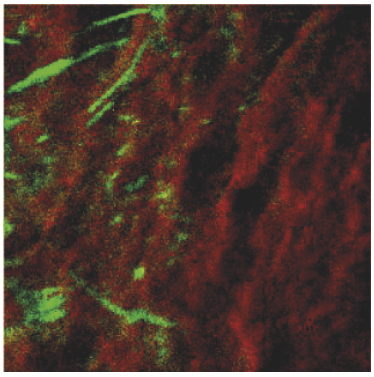

Figure 26. TOF-SIMS-generated image of cholesterol crystals in a human coronary artery intima. Cholesterol crystals are depicted in green. The phospholipids in intima are depicted in red. Each side of the image is 101 µm.

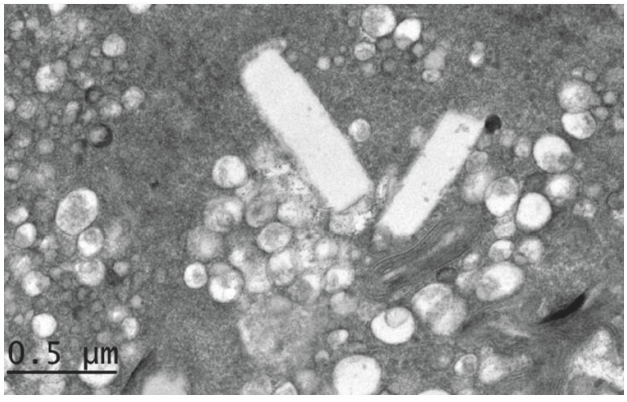

Figure 27. A transmission electron micrograph of human atherosclerotic carotid artery plaque. Atherosclerotic carotic artery plaques contained extracellular cholesterol crystals, multilamellar bodies and extracellular lipid particles.

In electron microscopic images of carotid atherosclerotic lesions, cholesterol crystals were found to be associated with extracellular lipid droplets (Figure 28; Study III, Figure 1), suggesting that the lipid particles indeed may grow from the lipid droplets. In the Figure 28 cholesterol crystals having different shapes can be seen: part of the crystals are needle-like and part of them are sheet-like, suggesting that the observed cholesterol crystals were in different phases of development. Cholesterol crystals are nucleated when a sufficient concentration is achieved, in other words, when the concentration exeeds the limit of solubility**.** The critical concentration of cholesterol both *in vitro* and in an atherosclerotic plaque is approximately 30 % of total lipid, which includes cholesterol, CE, and phospholipid (31.5 %, 47.2 %, and 15.3 %, respectively)(Katz, Shipley, and Small, 1976). Cholesterol crystal nucleation has been studied extensively in the context of bile, and in these studies a cholesterol crystal-growing process was described by Konikoff and co-workers (Konikoff et al., 1992). According to their studies, cholesterol crystals are needle-like in the early stage and only in the late stage, the crystals become sheet-like.

Unesterified cholesterol can be detected in atherosclerotic plaques with filipin stainings (Kruth, 1984), and also cholesterol crystals are found in the same areas in the arteries, and also in bioprosthetic aortic valves (Bocan, Schifani, and Guyton, 1986; Price et al., 2007). Murine models show that unesterified cholesterol is found in foam cells that have taken up unesterified cholesterol as re-esterification has decreased (Maor and Aviram, 1994) and that crystals form inside the lipid droplets (Kellner-Weibel et al., 1999) and lysosomes (Tangirala et al., 1994). Unesterified cholesterol can also be formed in tissues extracellularly from lipoproteins by lipolytic activity of LAL that is secreted by macrophages (Hakala et al., 2003) residing in the atherosclerotic plaque to the pericellular space. Cholesterol crystals also form inside macrophages (Klinkner et al., 1995; Pasquinelli et al., 1989; Tangirala et al., 1994), where LAL is active in the lysosomes and intralysosomal concentration of unesterified cholesterol can exeed the required 30 %.

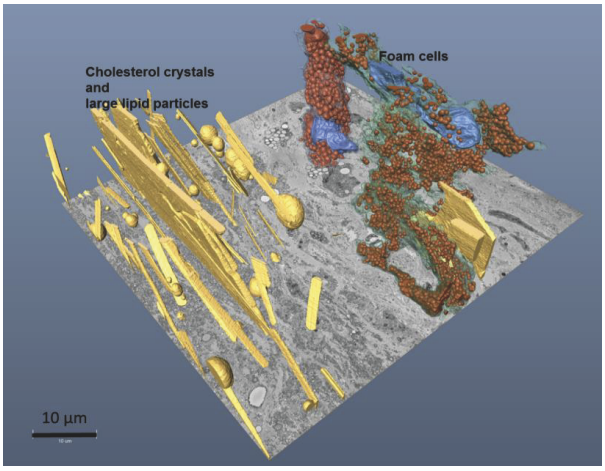

Figure 28. Three-dimensional EM of a carotid artery intima. Left cholesterol crystals and extracellular lipid droplets from which the crystals seem to grow out (yellow structures). On the right two foam cells, one of which surrounds a large cholesterol crystal. Blue structures are foam cell nuclei. Foam cells are filled with lipid droplets (maroon).

The cholesterol crystals imaged in a carotid artery intima with three-dimensional EM had reached the size of foam cells and as they formed large sheets in the tissue, they could even act as barriers to create distinct compartments (Figure 28). For the three-dimensional EM images, a sample of a carotid artery lesion was imaged with a serial block face-SEM, and the images, 40 nm apart, were stacked using a software. The

objects were semi-automatically selected from each layer, again combined with a software (Belevich et al., 2016), and given pseudo colors to bring out outlines of cells, crystals, and lipid droplets. Macrophages avidly phagocytoce cholesterol crystals (Rajamäki et al., 2010), and in the three-dimensional EM image, a macrophage was seen surrounding a large cholesterol crystal (Figure 28). Phagocytoced cholesterol crystals have been shown to activate the inflammasome both in mice and in human monocyte-derived macrophages (Duewell et al., 2010; Rajamäki et al., 2010).

Small extracellular lipid particles were also three-dimensionally imaged by electron tomography (ET) (Study III, Figure 1E and 1F) For ET, a 250-nm-section 250 nm section was imaged from 1 degree intervals. From the generated images, objects were manually traced and combined with a software. In the image, extracellular lipid droplets were seen to be aggregated and in a process of fusion in the intima. Lipoprotein-derived lipid particles in the arterial intima will aggregate and fuse after modifications combining oxidation, lipolysis and proteolysis as shown by in vitro experiments by Pentikäinen and co-workers and Öörni and co-workers reviewed (Pentikäinen, Lehtonen, and Kovanen, 1996; Öörni et al., 2000). Previously, fusing lipoprotein particles have been seen in experimental set-ups, such as in experiment of Nievelstein and co-workers, who injected LDL into rabbit circulation and after two hours the lipoprotein particles were seen aggregated and some particles seemed to be in the process of fusing (Nievelstein et al., 1991). In those images, however, the fusing of lipid particle membranes could not be specifically detected, as it is possible in the ET.

Protein composition of the extracellular lipid particles

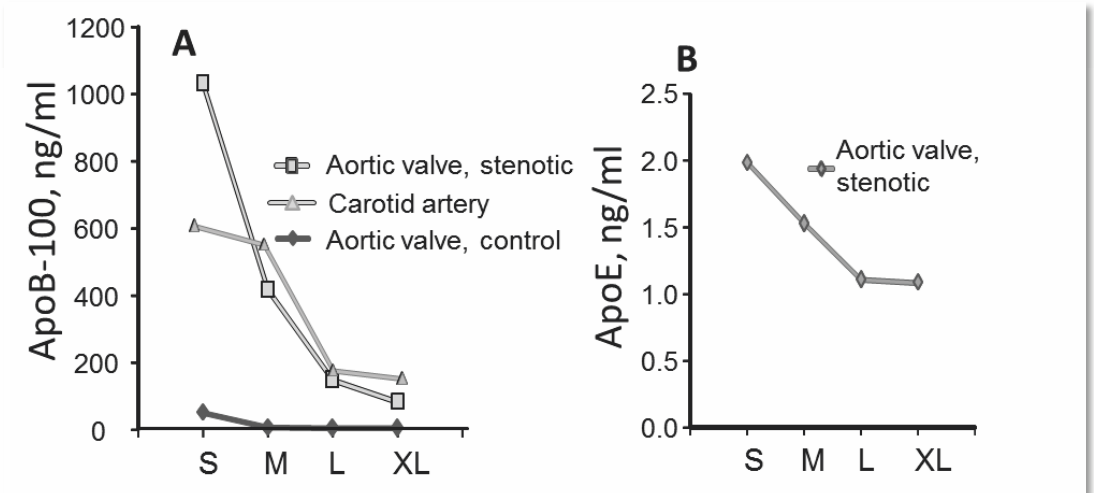

Figure 29. ApoB-100 and apoE-concentrations of aortic valve and carotid artery extracellular lipid particles. ApoB-100 and apoE-concentrations were measured with ELISA. ApoE-concentrations are measured from the same stenotic valve leaflets as the apoB-100-concentrations.

Traditionally, it has been suggested that the lipid pool of the arterial atherosclerotic lesions is composed particularly of lipid droplets that have been derived from dead foam cells. This may be accurate in the case of very advanced atherosclerotic lesions. Here, the protein compositions of the extracellular lipid particles were analyzed with ELISA-methods (Figure 29, Figure 30 A), Western blot and protein mass spectrometry

(Study III, Supplemental Figure III, Supplemental Table 1). The main protein in the extracellular lipid particles both in the aortic valves and in carotic arteries was found to be apoB-100 (Figure 29, Figure 30A). ApoB-48, the main protein component of the chylomicrons, is an intestinal splice variant from apoB-100, and comprises of 48 % of N-terminal amino acid sequence of apoB-100 (Chen et al., 1987), and hence, with the mass spectrometry, in which the proteins fragment extensively due to the trypsin treatment, apoB-48 is recognized only as a peptide or fragment of apoB-100. A pool that contained carotid artery extracellular lipid particles from several different patients, contained, in addition to apoB-100, small exchangeable apolipoproteins: apoA-I, apoE, ApoC-I, II, and III, apoM, apoD, and very small intensities of apoA-IV. ApoB-48 could not be recognized separately from apoB-100. The delipidated isolate of carotid artery extracellular lipid particles contained also components of the complement system. Complement components C3, C4b, and C9 were recognized in the delipidated protein component of the extracellular lipid particles. C3 is a component of the alternative pathway, C4b belongs to the classical pathway, and C9 is a component of the membrane attact complex and they have been suggested/show to be important in atherosclerosis (Oksjoki, Kovanen, and Pentikainen, 2003). In an atherosclerosis model, modified LDL is found to induce C3 secretion from human THP-1 macrophages *in vitro* (Mogilenko et al., 2012). A complement component downstream of C3, C3a, has been found to induce secretion of monocyte chemoattractant protein -1, IL-6, and IL-8 from myofibroblasts in stenotic aortic valves (Helske et al., 2008). In addition to the complement components, the isolate contained also several immunoglobulin species. As these proteins had floated with the lipid particles, it is possible that they had been parts of both complement component-containing immune complexes (Ylä-Herttuala et al., 1994) or bound to components of the modified lipid particles (Torzewski and Bhakdi, 2013) These immune complexes are avidly taken up by macrophages, and, depending on the immunoglobulin species, they could stimulate immunocompetent cells (Mantovani, Rabinovitch, and Nussenzweig, 1972).

Structural alterations of apolipoproteins

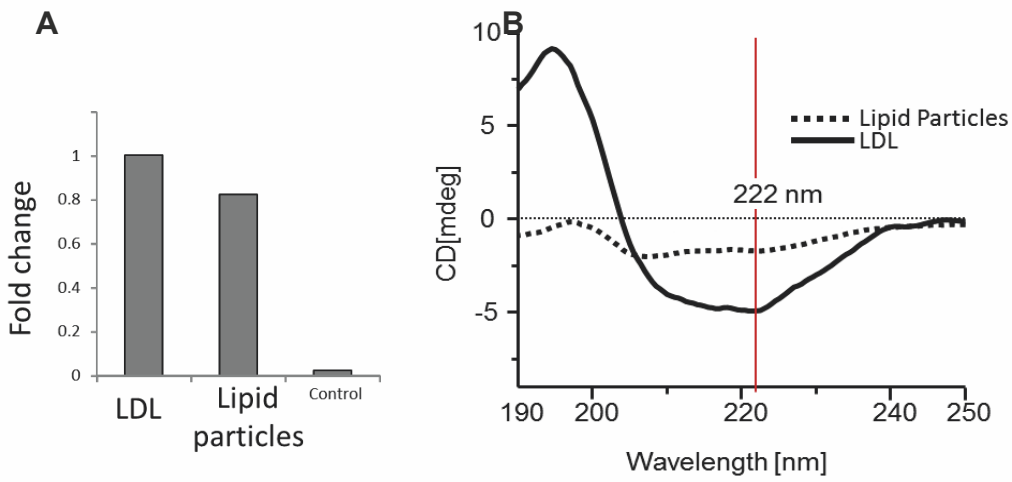

Figure 30 Circular dichroism analysis of the extracellular lipid particles and LDL. A. Direct apoB-100 -ELISA of LDL and the extracellular lipid particles, both 5 µg/ml in total protein content. The ELISA-analysis shows that apoB-100 is a prominent protein in the extracellular particles. B. The secondary structure of the apoB-100-containing extracellular lipid particles is dramatically different from the structure of plasma LDL. The change in α-helicality can be detected at 222 nm.

ApoB-100 of plasma LDL has in its native state α -helix, β-sheet, and random structures (Chen et al., 1987; Law et al., 1986; Scott et al., 1987), and there are two proteoglycan binding sites (aa 3359-3367 (site B) and aa 3147-3157 (site A) (Boren et al., 1998b), of which the former functions also as a LDL receptor-binding site (Boren et al., 1998a). Circular dichroism analysis of the carotid extracellular lipid particles indicated that the protein conformation of them was significantly different from that of LDL particles. Thus 35 % of apoB in LDL particles was found to be α-helical (Figure 30 A; Study III, Figure 2.), which is well in accordance with studies by Chen and co-workers, who reported the the α-helix content of apoB-100 of native LDL is approximately 40 % (Chen 1986). However, the α-helical content of the extracellular particle isolate was approximately only 4 % and the secondary structure of the proteins was almost totally deteriorated into random coil. Such result could be due to proteolytic degradation of the particles, but also lipolysis can affect the protein structure of apoB-100. Sneck and co-workers (Sneck et al., 2012) studied the effects of SMase and acid pH on the conformation of apoB-100, and their experiments showed that lipolysis by itself can significantly alter its conformational structure. They showed that apoB-100 lost most of its α-helical structure, which, actually, had transformed into β-sheets. In amyloid fibrils, the proteins lose their native conformation. The small exchangeable apolipoproteins, as well as apoB-100, are highly susceptible to amyloidogenesis due to their hydrophobic helix-content, and indeed, apoE, apoA-I, apoA-IV, apoC-II, and apoC-III have been reported to form amyloid fibril (Das and Gursky, 2015). ApoB-100 is very stable in its native state (Das and Gursky, 2015), but apoB-100 in electronegative LDL has been found to form amyloid fibrils (Parasassi et al., 2008). Also hydrolysis by PLA_2 increases instability of apoB-100 in an experimental set up (Jayaraman, Gantz, and Gursky, 2011). Therefore it is not impossible that the extracellular particle isolate would contain amyloid, as apart from apoB-100, the extracellular lipid particle pool was found to contain several different apolipoproteins (apoE, apoC-I-III, and apoA-I) all of them having a high quantity α-helical content. According to ELISA-analysis, apoB-100 it the most abundant protein component in the extracellular particle preparate (Figure 30 B). ApoB-100 is the most abundant protein, and it comprises 95 % of LDL protein (Karlsson et al., 2005), and it remains to be reliably shown that apoB-100 actually forms amyloid fibrils. Also the intracellular lipid droplets carry proteins on their surface, (Guo et al., 2009) some of which, such as several members of the PAT-protein family, are also α-helical by structure (Bickel, Tansey, and Welte, 2009), but, in the present study the proportion of intracellular lipid particle proteins was very low in the isolates from both aortic valves and from carotid arteries. Perilipin-2 was not detected from the stenotic aortic valves perilipin-2 with Western blot (Study II, Figure 4). When the extracellular lipid particles from the carotid arteries were analyzed, the mass protein spectrometry analysis did not detected any intracellular lipid droplet proteins among the first one hundred recognized proteins Study III, Supplemental Table I.

Foam cells in the intima

In order to study the effect of the extracellular lipid particles on foam cell formation, monocyte-derived primary human macrophages were treated with LDL, VLDL, cholesterol crystals and extracellular lipid particles isolated from human carotid artery plaques (Figure 30; Study III, Figure 6). The macrophages formed foam cells when treated with VLDL, cholesterol crystals, and extracellular lipid particles, however the lipid composition of the cells were different (Figure 31). The macrophages that had taken up cholesterol crystals were rich in CEs, while the cells that had taken up VLDL were rich in TAGs. Interestingly, the macrophages that had taken up extracellular lipid particles were enriched with TAGs and unesterified cholesterol over CEs, having similar lipid composition as the cells that had taken up VLDL. Interestingly, the lipid composition of the carotid extracellular lipid particles (Figure 22) seemed to be very different from that of VLDL (Figure 23). In this case one explanation would be lipid composition differences between different carotid artery donors and thus differences between different particle batches.

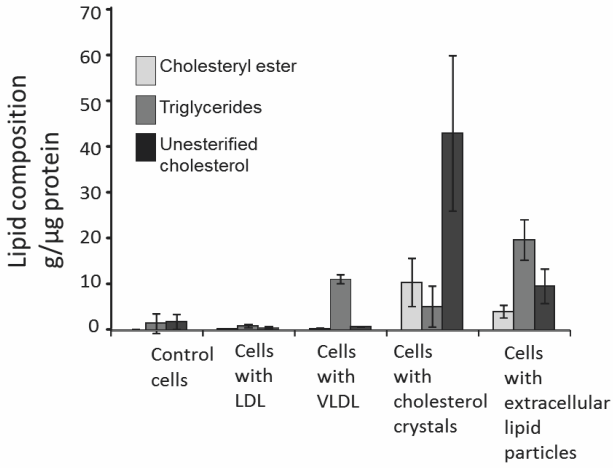

Figure 31. Foam cell lipid content of human primary macrophages treated with LDL, VLDL, cholesterol crystals, and extracellular lipid particles. High concentration of unesterified cholesterol in the cells treated with cholesterol crystals is artefact from cholesterol crystals attached on the cells.

Macrophages take up oxidized lipoproteins avidly by receptors like CD36 (Endemann et al., 1993) or **L**ectin-like **Ox**idized low-density lipoprotein receptor-1 (LOX-1)(Kume and Kita, 2001), and become lipid-filled foam cells. Oxidized LDL stimulates endothelial cells to produce MMP-1, a metalloprotease with a collagenase acitivity. MMP-1 promotes neovascularization of the atherosclerotic plaques, as the protease is able to clear a path for novel vasculature (Huang, Mironova, and Lopes-Virella, 1999; Huang et al., 2001). Oxidized LDL has also been shown to stimulate VEGF-production in monocytes, also a crucial factor in neovascularization (Inoue et al., 2001). In lysosomes of human leukemia cell line THP-1 cells oxidized LDL can inhibit CE hydrolysis, and thus accelerate accumulation of CE and free cholesterol in lysosomes of macrophages (Yancey and Jerome, 2001). Thus, uptake of oxLDL by macrophages results in lipid-filled macrophages, in which, insted of the cytoplasmic accumulation of CE-filled lipid droplets, CE also accumulates in the lysosomes. However, the relative proportion of lysosomal accumulation of CE strongly depends on the degree of LDL oxidation; thus, mildly oxidized LDL does not lead to significant lysosomal accumulation of CE or free cholesterol in macrophages. Murine macrophages metabolize oxidized LDL differently, in that oxidized LDL accumulates in mouse macrophages only transiently (Yancey and Jerome, 2001). In mouse macrophages, oxidized LDL is known to activate the inflammasome (Duewell et al., 2010) and thus trigger the macrophages to secrete proinflammatory cytokines.

While CEs accumulate in the foam cells, TAGs do not usually accumulate. In macrophages that have been isolated from atherosclerotic lesions, only 15 % of the neutral lipids is TAG (Mattsson et al., 1993). In hypoxia, however, β-oxidation in the macrophages decreases and accumulation of TAGs can occur (Boström et al., 2006). Hypoxia in the atherosclerotic plaque is, however, a transient stage, as neovessels grow in increasing numbers in the advanced stage plaques and provide oxygen to the hypoxic areas (Study I). In the TOF-SIMS analysis of the coronary artery sections (Study I), the spatial distribution of TAGs was found to differ from the spatial distribution of cholesterol and CEs, as TAGs were found near the endothelium or near the endothelia of the neovascular capillaries. From MS-images it was not possible to distinguish whether TAGs were on the luminal side of endothelial cells or whether they had crossed the endothelium. TAGs may have access to the intima with VLDL or VLDL-remnants, or in chylomicron-remnants. Lipoprotein particles smaller than 75 nm can enter the subendothelial space (Nordestgaard and Zilversmit, 1988) and the entrance can be enhanced through endothelial damage (Frank and Fogelman, 1989) by free fatty acids released by endothelial cell-associated LPL (Pentikäinen et al., 2002).

Inflammasome activation by extracellular lipid particles

As cholesterols crystals are an established activator of NLRP3-inflammasome in human monocyte-derived macrophages (Rajamäki et al., 2010), we were prompted to test whether the estracellular lipid particles from human carotid artery lesions would also activate the inflammasome and, also by this way, be an initiating factor in atherosclerosis. The extracellular lipid particles were indeed found to induce IL-1β secretion from macrophages *in vitro* in human monocyte-derive macrophages that were primed with lipopolysaccharide (LPS) as a first stimulus. IL-1β, in turn, is capable in inducing SMase secretion from macrophages (Marathe 1998). The mRNA-expression of NLRP3 pikes already at 1 hour incubation with cholesterol crystals in LPS-primed cells. (Rajamäki et al., 2010). Interestingly, Rajamäki and co-workers showed that cholesterol crystals activate the IL-1β secretion slowly: the high point of cholesterol crystal-induced caspase-1 mRNA expression in LPS-primed cells is at 9 hour time point (Rajamäki et al., 2010). Slow production of IL-1β was also detected when the primary human monocyte-derived macrophages, or THP-1 cell-line macrophages, were treated with the extracellular lipid particles. Thus, an overnight incubation of the THP-1 cells in vitro was required for remarkable inflammasome activation, as measured by appearance of specs in the cells, and for IL-1β secretion. The IL-1β -secretion that was followed by macrophage incubation with the extracellular lipid particles was almost 2-fold compared with the activation by cholesterol crystals. While cholesterol crystals were detected in the extracellular lipid particles (Study III, Figure 3), the particles isolated also contained lipid droplets with features of modified lipoproteins, and thus the cytokine secretion was likely induced by the combination of several different activators.

To find, which component of the extracellular lipid particles was the culprit for inflammasome activation, LDLwas treated with SMase, PLA_2, and α-chymotrypsin, or oxidized by $CuSO_4$ and thereafter incubated with or without LAL (Study III, Figure 5B). Only such LDL particles in which surface hydrolysis with PLA_2 was combined with the core hydrolysis with LAL, were able to induce IL-1β secretion that was at least as strong as activation with cholesterol crystals. Interestingly, and somewhat surprisingly, oxidized LDL did not trigger IL-1β secretion from human monocyte-derived macrophages, while in previous experiments by Duewell and co-workers in a mouse model, it was a strong activator of inflammasome (Duewell et al., 2010). None of the tested activators could induce IL-1β secretion without LPS-priming (Study III, Figure 5B). As NLRP3-inflammasome activation requires a two-step activation (Latz, Xiao, and Stutz, 2013), it suggests that none of the LDL hydrolysis products used for activation were able to prime the inflammasome activation pathway by themselves. On the contrary, the activation always needed a preceeding lipopolysaccharide-treatment as a priming step.

To test, whether IL-1β -secretion was truly due to inflammasome activation, the effect of cytochalasin D and caspase-1-inhibitor Z-YVAD on IL-1β -secretion was examined (Study III, Figure 5D). Cytochalasin D prevents the uptake of modified lipid particles, and caspase-1 is inhibited with Z-YVAD, that prevents autoproteolysis of caspase-1 to mature caspase-1 and thus prevents IL-1β secretion from the cells (Guey et al., 2014). Both inhibitors were found to prevent IL-1β secretion from the primary monocyte-derived macrophages, which were stimulated with estracellular lipid particles and with LDL hydrolyzed with the combination of PLA_2 and LAL. As caspase-1 proteolysis is triggered by NLRP3-inflammasome activation and the mature protease is required for IL-1β secretion, prevention of the IL-1β secretion with a caspase inhibitor suggests that the extracellular lipid particles do indeed activate the NLRP3 inflammasome. Also, uptake of the extracellular lipid particles appears to be required for activation of the inflammasome in macrophages.

One component of the extracellular matrix, cholesterol crystals, has been shown to be a potent activator of inflammasome both in murine models and in human macrophages (Duewell et al., 2010; Rajamäki et al.,

2010). In Study III, the extracellular lipid particles isolated from carotid arteries were shown to induce inflammasome activation inflammasome in primary human monocyte-derived macrophages in vitro. As the accumulated lipid particles in the aortic valves closely resembled the particles isolated from the carotid arteries in their lipid composition, size distribution, and protein composition, it could be expected that similar phenomena, such as lipolysis, proteolysis, and oxidation of the retained lipoproteins would take place in both tissues. Both cholesterol crystals (Rajamäki et al., 2010) and calcium crystals (Wen et al., 2013) have been found to activate the inflammasome and thus increase the severity of atherosclerosis, and, moreover, inflammasome activation has been also shown to increase calcific nodule formation in smooth muscle cells (Wen et al., 2013). Taken together, not only cholesterol crystals, but also the lipoproteins that have been lipolyzed with PLA_2 and LAL can activate the inflammasome and thus induce macrophages to produce IL-1β. On the other hand, SMase or proteolysis with α-chymotrypsin alone or in combination with LAL did not produce an inflammasome activation to a similar extent. The most potent activator of inflammasome in human atherosclerotic lesions and the exact mechanism of such activation are still to be recognized.

Concluding remarks

The aims of this thesis were to examine how the lipids, such as are known to take place in the atherosclerotic intima, are spatially arranged in various phases of atherosclerosis, to image the lipid particles and cholesterol crystals, to isolate and characterize lipids from the extracellular compartment of aortic valve and atherosclerotic arteries with various state-of-the-art methods, and to examine whether the extracellular lipid particles affect inflammation and cytokine relase in cells relevant for atherosclerosis.

1. The lipids in the coronary arteries were found to be spatially arranged in the intima. In normal intima the detected lipids are cell plasma membrane lipids that consist of phospholipids and unesterified cholesterol. In the atheroma-stage and in the advanced-type lesions of atherosclerosis, most of the lipid was composed of CEs and unesterified cholesterol, which lipids accumulated deep in the intima. Interestingly, TAGs were very seldom found to co-localize with cholesterol of either type, rather they localized only periendothelially either near the luminal endothelium or near the endothelia of neovascular microvessels. The differential spatial arrangement of the lipids start already in the fatty streak stage and it was the most prominent in the distributions of cholesterol and TAGs. The newly introduced ratio of two cholesterol ions (385/369) provides a way to distinguish signals of esterified cholesterol from unesterified cholesterol even if the molecular ions have fractured in the analysis.

2. The extracellular lipid particles were isolated and characterized. The lipid composition of the lipid particles isolated from either aortic valves or from atherosclerotic arterial intima was different from plasma lipoproteins. Free fatty acid contents were increased, as was the proportion of unesterified cholesterolThe proportion of PC was decreased comparet to plasma LDL. This degradation of the surface and core lipids of the lipid particles was most likely due to action of both lipolytic and proteolytic enzymes. The extracellular lipid particles were found to contain several apolipoproteins, such as apoB-100, which was the main protein, and also apoE, apoC-I, II, and III, and apoA-I and IV. Also components of complement and immunoglobulins were found. The conformation of the protein moiety of the lipid particles was dramatically different from that of the plasma lipoproteins, as the α-helix-content and organized secondary structure of the proteins were only fraction of that found in plasma lipoproteins. In stenotic aortic valves, lipid composition had remarkable individual differences between the patients. Similar individual differences in should be analyzed from the carotid artery plaques. These individual differences in lipid composition would perhaps partly expain also why atherosclerotic plaques develop into stable or unstable plaques.

3. The extracellular lipid compartment isa component of atherosclerosis which actively contributes to inflammation. The extracellular lipid is composed of lipoprotein-derived lipid particles that have been modified by lipolytic and proteolytic enzymes. Also a minor proportion of apoptotic foam cell-derived lipid droplets can be found extracellularly. In vitro cholesterol crystals can be generated from LDL and VLDL a result from weakening the surface and hydrolysing the core lipids. The unesterified cholesterol derived from the extracellular lipid particles canserve as a source of cholesterol crystals. These crystals combined to the lipid particles are able to activate a multiprotein complex called the inflammasome, to produce pro-inflammatory cytokines, such as IL-1β. In addition to the crystals, other possible components of lipid particles that are contributing to activation of the inflammasome. The most potent activation was experimentally archieved by phospholipid hydrolysis products LPCs and free fatty acids, but the overall activation is likely resulted from a combination of modifications.

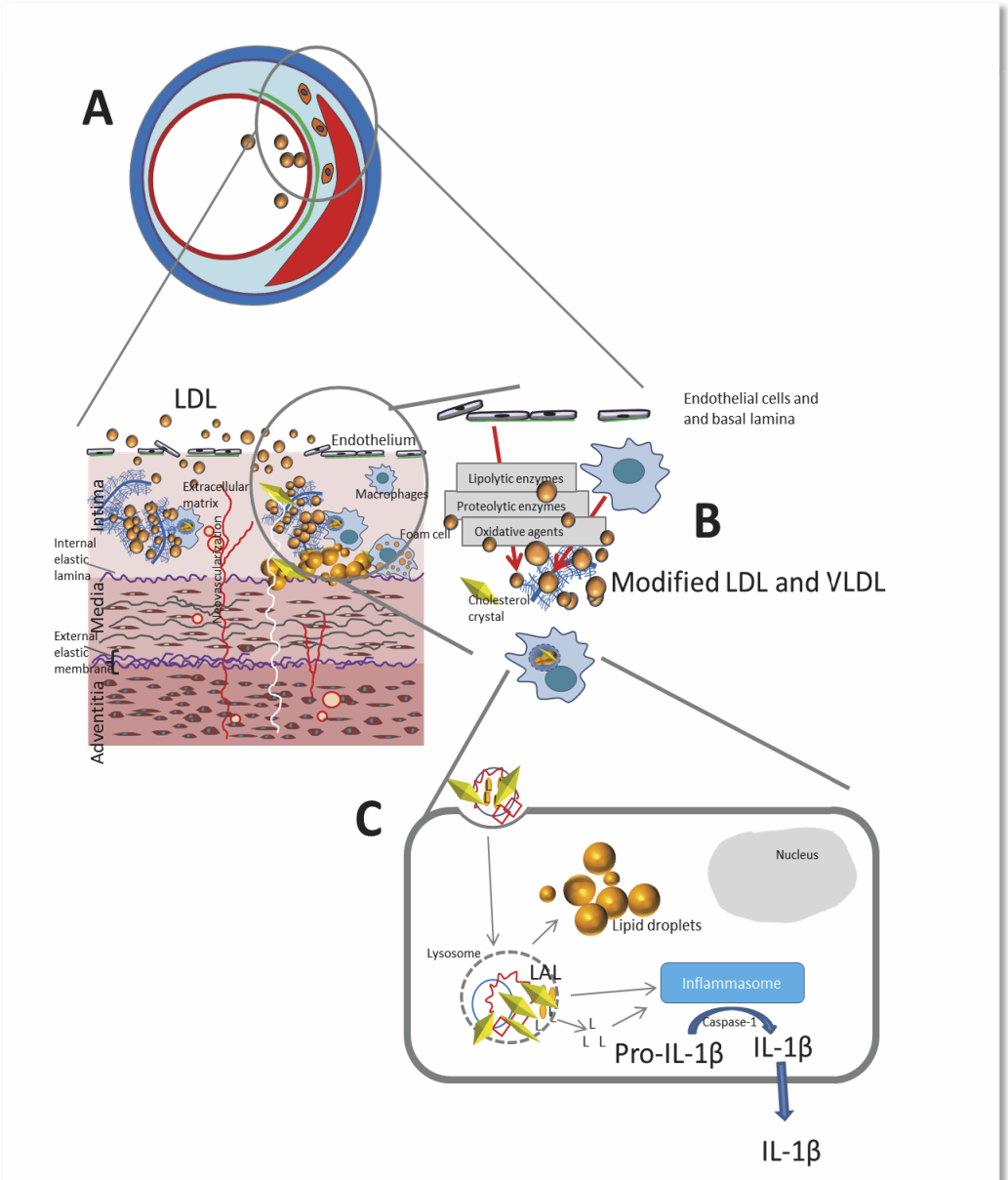

Figure 32. The extracellular lipid particles, their accumulation, and their effects. Plasma LDL enters the intima, attaches to the extracellular matrix proteoglycans, and accumulates near the internal elastic lamina. The retained LDL is modified by lipolytic and proteolytic enzymes, and by oxidation, and is subsequently aggregated and fused, with ensuing formation of extracellular lipid particles, and the cholesterol in in these particles is crystallized. The extracellular lipid particles and crystals are taken up by macrophages which induces inflammasome activation and the cells produce inflammatory cytokines.

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